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The Phenotype and Genotype of Children with Newly Diagnosed Type 1 Diabetes in Relation to Family History of Type 1 Diabetes and Other Autoimmune Diseases

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The phenotype and genotype of children with newly diagnosed type 1 diabetes in relation to family history of type 1 diabetes and other autoimmune diseases

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ACADEMIC DISSERTATION

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Abstract

Background

Type 1 diabetes (T1D) is an immune-mediated disease that affects ~0.7% of children in Finland. Its incidence in Finland is the highest worldwide. The disease starts with an asymptomatic period with an ongoing immune-mediated destruction of β-cells and detectable autoantibodies against islet antigens. This process leads to hyperglycemia and eventually clinical symptoms when insulin secretion is no longer sufficient. The proposed etiology of T1D is both genetic and environmental. Many of these etiological factors are shared between other autoimmune diseases (AIDs) and, accordingly, these diseases co-occur in patients with T1D and their relatives. This thesis aims at characterizing the frequency of additional autoimmunity in Finnish children under the age of 15 years with newly diagnosed T1D and in their extended family members, as well as characterizing the effects of this additional autoimmunity on clinical, metabolic, and genetic markers, and T1D autoantibodies.

Subjects and methods

The subjects for this thesis are participants of the Finnish Pediatric Diabetes Register and Sample Repository. This is a nationwide register inviting participation of all newly diagnosed patients with T1D from all pediatric units in Finland. Over 90% of the patients and families participate in the Register. With the help of their medical team, the participating families fill in questionnaires on the clinical parameters, family characteristics and diabetes and other diseases of the index child and family members. Data on these diseases is collected with open questions, but examples such as celiac disease (CD), thyroid dysfunction, adrenal dysfunction, rheumatoid arthritis, multiple sclerosis, pernicious anemia and systemic lupus erythematosus are given. Approximately 70% also give blood samples for the Sample Repository to study childhood diabetes and its comorbidities. This thesis analyses data from the time of diagnosis on 2245 children diagnosed with T1D since the beginning of the Register in January 2002 up to November 2009. The mean age at diagnosis of T1D for the study population is 7.9 years and the majority are boys (57.1%). For a subset of the cases, follow-up data is available.

Results

Additional AIDs were reported by 1.6% children at diagnosis and by 3.2% after a median eight years of follow-up. After follow-up, CD was reported by 1.5%, autoimmune thyroid disease by 1.3%, rheumatoid disease by 0.3% and other AIDs by 0.2% of the 2245 children. More than 20% of the families reported first- and/or second-degree relatives with T1D, and over a third, relatives with other AIDs. Fathers were more often affected by T1D compared to mothers (6 vs. 3%), whereas mothers were more often affected by other AIDs (10 vs. 4%). Girls had more often T1D affected paternal and boys T1D affected maternal second-degree relatives. At T1D diagnosis, 5% of the index children and 3% of their relatives had tissue transglutaminase autoantibodies (anti-tTG) related to CD. Transient anti-tTG not developing to CD seemed more common among children with T1D than among their relatives. The HLA-DR3-DQ2 haplotype was associated with CD autoimmunity and the HLA-DR4-DQ8 haplotype with familial T1D. The children with AIDs other than CD had neutral or protective T1D related HLA genotypes conspicuously often. Also, non-HLA loci were shown to contribute to the clustering of AIDs in children with multiple AIDs and in autoimmune families. Familial T1D, even with only second-degree relatives affected, leads to less severe onset of T1D in the index child. There was some evidence for a milder onset of T1D in children with additional AIDs.

Conclusions

This thesis provides current estimates of the frequency of additional autoimmunity in Finnish children with newly diagnosed T1D and in their relatives: at diagnosis 1.6% of children had additional AIDs, over 20% extended family members with T1D and over 30% extended family members with other AIDs. These figures are in line with those reported previously internationally and in Finland. Differences in genetic etiology was implicated behind different phenotypes of clustering autoimmunity; familial T1D associated with DR4-DQ8 and T1D with CD autoimmunity with DR3-DQ2. Definitive associations of clustered autoimmunity with for example certain islet autoantibodies were not evident. Novel discoveries were the milder clinical onset of T1D in familial T1D even if only second-degree relatives were affected (readily explained by the increased awareness of the disease in these families), and the gender difference of girls having paternal and boys maternal second-degree relatives affected by T1D. This gender difference, transient anti-tTG among children with T1D at diagnosis, and the reported candidate non-HLA SNPs for clustered autoimmunity require validation by further studies.

List of original publications

This thesis is based on the following publications:

I Parkkola A, Härkönen T, Ryhänen SJ, Ilonen J, Knip M, and the Finnish Pediatric Diabetes Register. Extended family history of type 1 diabetes and phenotype and genotype of newly diagnosed children. *Diabetes Care* 2013; 36:348-354.

II Parkkola A, Härkönen T, Ryhänen SJ, Ilonen J, Knip M, and the Finnish Pediatric Diabetes Register. Extended family history of autoimmune diseases and phenotype and genotype of children with newly diagnosed type 1 diabetes. *Eur J Endocrinol* 2013;169:171-8.

III Parkkola A, Härkönen T, Ryhänen SJ, Raivo U, Ilonen J, Knip M, and the Finnish Pediatric Diabetes Register. Transglutaminase antibodies and celiac disease in children with type 1 diabetes and in their family members. *Pediatr Diabetes* 2017;0:1-9. https://doi.org/10.10111/pedi.12563

IV Parkkola A, Laine AP, Karhunen M, Härkönen T, Ryhänen SJ, Ilonen J, Knip M, and the Finnish Pediatric Diabetes Register. HLA and non-HLA genes and familial predisposition to autoimmune diseases in families with a child affected by type 1 diabetes. *PLOS ONE* 2017;12:e0188402

The publications are referred to in the text by their Roman numerals.

Abbreviations

ACA	Adrenocortical antibodies
AID	Autoimmune disease
AIT	Autoimmune thyroiditis
Anti-tTG	Tissue transglutaminase antibodies
BMI	Body mass index
CD	Celiac disease
CHR	Chromosome
CTLA-4	Gene encoding cytotoxic T lymphocyte antigen 4
Dg	Diagnosis
EMA	Endomysial antibodies
ESPGHAN	The European Society for Paediatric Gastroenterology, Hepatology and Nutrition
FDR	False discovery rate
FUT2	Gene encoding fucosyltransferase 2
GAD	Glutamic acid decarboxylase
GADA	Autoantibodies against the 65 kD isoform of glutamic acid decarboxylase
GWAS	Genome-wide association study
HLA	Human leucocyte antigen
IAA	Insulin autoantibodies
IA-2A	Autoantibodies against islet antigen 2 protein
ICA	Islet cell antibodies
IFIH1	Gene encoding interferon induced helicase C domain 1
Ig	Immunoglobulin
INS	Gene encoding insulin
JDFU	Juvenile diabetes foundation units
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
MAF	Minor allele frequency
MHC	Major histocompatibility complex
MODY	Maturity onset diabetes of the young
MS	Multiple sclerosis
NOD	Non-obese diabetic
PCA	Parietal cell antibodies

PTPN22	Gene encoding protein tyrosine phosphatase, non-receptor type 22
OR	Odds ratio
RU	Relative units
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
T1D	Type 1 diabetes
tTG	Tissue transglutaminase
Tregs	T regulatory cells
Yrs	years
ZnT8A	Zinc transporter 8 autoantibodies

Introduction

Type 1 diabetes (T1D) is a chronic immune-mediated disease caused by destruction of the insulin producing β-cells of the pancreas. It is characterized by a prodrome period when autoantibodies to different β-cell antigens can be detected in the peripheral circulation as a sign of ongoing β-cell destruction although glucose homeostasis is still accurately maintained. When enough of the β-cells are destroyed and the insulin producing capacity is no longer sufficient, the patient becomes permanently dependent of exogenous insulin.

In addition to vigilant glucose monitoring and insulin injections to maintain good metabolic control, patients with type 1 diabetes have a significantly increased mortality compared to the general population [1, 2]. Additionally, T1D puts a strain on the society and health care system with substantial cost. Thus, studies on the etiology, pathogenesis, natural course, and prevention of T1D are well justified.

Increased understanding the etiology and pathogenesis of the immune reaction in T1D is crucial to efforts on trying to find effective prevention strategies for the disease. As this knowledge has accumulated, we have come to understand that in addition to mechanisms unique to the T1D autoimmune reaction, many of the proposed risk factors and pathways are shared with other autoimmune diseases (AIDs). Accordingly, patients with T1D and their relatives are at an increased risk not only for T1D but for other AIDs as well. For example, same HLA class II risk genotypes predispose for T1D and celiac disease (CD).

This thesis aims at characterizing some of the factors associated with T1D pathogenesis and their relation to a more general propensity to autoimmunity.

Review of the literature

Epidemiology of type 1 diabetes

T1D was relatively uncommon until the 1950s when the incidence started to increase simultaneously in different parts of the world. Since then approximately 3% yearly linear increase in the incidence of T1D has been observed [3-5]. In 1999 it was estimated that the T1D incidence would increase by 40% from year 1998 to 2010 [4]. According to these estimates the incidence in Finland would have been 50/100 000 by 2010. The incidence reached 64/100 000 already in 2005 [6], however. The greatest increase in incidence has been among the youngest age group (0-5-year-olds) and in the countries with lower disease incidence previously [3, 5, 6]. Recently, an encouraging development has been described; reports of a plateau or even decrease in incidence have been seen around Scandinavia and Finland [7, 8].

Globally, T1D incidence is the highest in Finland and Sardinia. In general, the incidence is high in developed countries, and lower in countries with lower standard of living. The reasons for these differences are unknown, but supposedly related to differing environmental and genetic factors across the globe.

Pathogenesis of type 1 diabetes

T1D is an immune-mediated disease with destruction of insulin producing β-cells of the pancreatic islets. The strong association with the human leucocyte antigen (HLA) region, partial effects demonstrated by immunomodulatory treatments, co-occurrence with other autoimmune diseases, and the insulitis observed in patients with T1D are regarded as indications for autoimmune process causing T1D. However, the leucocyte infiltration in the pancreas is much less pronounced than for example in the non-obese diabetic (NOD) mouse or in affected tissues of other AIDs, e.g. rheumatoid arthritis or psoriasis [9, 10]. Similarly, the effect of immunomodulatory treatments is far from that seen in other AIDs. Accordingly, autoimmune origin of T1D – at least as a universal cause in all patients - has been challenged and the possibility of infections agents behind the disease has been raised. For the purpose of this thesis, however, T1D (with a range of other immune-mediated diseases) is grouped under AIDs.

T1D is caused by an interplay of genetic and environmental factors. Overwhelming majority of patients has a predisposing genotype in HLA and/or other risk regions. Only 5-10% of the genetically susceptible will develop the disease in their lifetime, however. Accordingly, environmental risk factors are crucial in disease development. It is believed that multiple precisely timed environmental stimuli are needed to first initiate the immune-mediated destruction of β-cells, and second, to keep the process progressing instead of restorating [11, 12].

Prediabetic disease process and humoral autoimmunity

The asymptomatic prediabetic period during which β-cell destruction progresses varies in length from only few months to more than a decade [13]. The clinical disease with symptoms related to hyperglycemia will not develop until 80-90% of β-cells have been destroyed, and any remaining functioning β-cells are thought to be destroyed within a few years from the diagnosis. This view has been challenged recently, however, with the notions that majority of patients (73%) after five years from the diagnosis of T1D still secreted low levels of insulin in response to a meal [14].

The destruction of β-cells is regarded as a T-cell mediated process with the humoral immune reaction being mostly secondary to the ongoing cell destruction. Nevertheless, autoantibodies to β-cell antigens can be detected and are used as a marker of ongoing β-cell destruction and in prediction of T1D. The major autoantibodies are islet cell antibodies (ICA), antibodies against glutamic acid carboxylase (GADA), insulin autoantibodies (IAA), autoantibodies against tyrosinphosphatase like protein/islet antigen 2 (IA-2A), and autoantibodies agaist zinc transporter 8 (ZnT8A). The schedule and order of appearance of different autoantibodies is highly variable [13]. One positive autoantibody is not necessarily a marker of significant risk for development of T1D, but the risk increases with increasing number of positive autoantibodies [15]. In a recent study, the risk to develop T1D by the age of 15-years was 0.4% for children without autoantibodies, 13% for children with one autoantibody, 62% for children with two autoantibodies, and 79% for children with three autoantibodies [16]. Risk factors associated with fast development of type 1 diabetes are the early appearance of autoantibodies (before the age of 3 years), the high-risk HLA genotype DR3-DQ2/DR4-DQ8, and female sex [16].

Islet cell autoantibodies (ICA)

ICA were the first T1D related autoantibody to be described [17, 18]. They are measured with an immunofluorescence method and thus measure immunoglobulin – mostly IgG – binding on multiple islet cell structures. Accordingly, ICA differ from other islet autoantibody modalities in that they are not specific to a single antigen but reflect reactivity to wider range of antigens. High levels of ICA reflect T1D related autoimmune destruction, but low levels of these autoantibodies are relatively common in relatives of T1D patients or general population and are thus assumed to represent non-progressive autoimmune reaction [19]. ICA are associated with younger age at onset, HLA-DR4-DQ8, and female gender [20]. At diagnosis, 84% of Finnish children with T1D are positive for ICA [21].

Insulin autoantibodies (IAA)

IAA [22] are prevalent especially in young children and appear often as the first autoantibody to be detected [23]. They associate with the HLA-DR4-DQ8 haplotype [24] as well as *INS* and *PTPN22* risk loci [25, 26]. As a first autoantibody to appear, IAA have a peak appearance before 2 years of age, and as a secondary autoantibody, IAA appear evenly over a wide age range [27]. In general, 44-92% of recent onset diabetic subjects [28], and 48-54% of Finnish children, have IAA at diagnosis of T1D [21, 29].

Glutamic acid decarboxylase autoantibodies (GADA)

Autoantibodies against the 65 isoform of glutamic acid decarboxylase were first described as autoantibodies against the 64K protein [30], which was later identified as GAD65 [31]. These autoantibodies are associated with older age, female gender and presence of the HLA-DR3-DQ2 haplotype [20, 32]. If GADA are the first autoantibody to appear, they appearance is widespread peaking between 3-5 years of age and decreasing thereafter, whereas as a secondary autoantibody they usually appear early after IAA [27]. GADA are not restricted to T1D but are occasionally seen in other autoimmune conditions. Accordingly, GADA are suggested to be related to general propensity to autoimmunity [24, 33, 34]. GAD protein is expressed also in tissues outside pancreas, for example central nervous system, as seen in stiff man syndrome. GADA

persist longer after diagnosis than other autoantibodies [35]. At diagnosis, 65-68% of Finnish children with T1D have GADA [21, 29].

Islet antigen 2 autoantibodies (IA-2A)

Protein tyrosin phosphatase family protein IA-2 [36, 37] and IA-2ß [38] are major autoantigens in T1D. Both are enzymatically inactive membrane spanning proteins, that function in the regulation of insulin secretion [39, 40].

These autoantibodies are associated with the HLA-DR4-DQ8 haplotype, and usually appear towards the end of the prediabetic process and are the most specific for the development of T1D [15]. IA-2A are rarely the first autoantibody to appear, but if they are, the process leads rapidly to clinical T1D [27]. Their predictive value for disease manifestation is very high, and they have been associated with rapid progression from islet autoimmunity to clinical disease [41]. An isolated IA-2A positivity has been proposed as a more aggressive phenomenon than isolated positivity to other autoantibodies [29]. At diagnosis, 75-79% of Finnish children with T1D have IA-2A [21, 29].

Zinc transporter 8 autoantibodies (ZnT8A)

Autoantibodies against zinc transporter 8 molecule are among the most recently characterised T1D related autoantibody modalities [42]. They were discovered after the recognition of of this molecule as a pancreas specific transporter [43]. In fact, ZnT8 is specific for β-cells and participates in insulin secretion and transports zinc into secretory granules for insulin storage in hexamers bound by zinc [44]. Over 60% of patients with T1D have ZnT8A [28] whereas for patients with other AIDs the proportion is 10% [42]. Also, the gene encoding ZnT8, *SLC30A8*, has been associated with T1D [45]. ZnT8A positivity is associated with older age, lower probability of diabetic ketoacidosis at diagnosis, lower probability of having the HLA-DR3/DR4 genotype, and presence of neutral DR13-DQB1*0604 haplotype [46]. In Finnish children with T1D, 63% had ZnT8A at diagnosis [46].

Etiology of type 1 diabetes

Genetics of type 1 diabetes

Traditionally, genes have been estimated to explain a major part of familial clustering of T1D. The concordance rate for monozygotic twins in Finland is 43% and for dizygotic twins 7% [47]. The HLA region is estimated to explain roughly 50% of the genetic predisposition to T1D [24] and to date over 50 other gene loci have been associated with T1D.

Human leucocyte antigen (HLA)

The most important genetic determinant of T1D risk with odds ratio (OR) 6-10 [48], is the HLA region in the short arm of chromosome 6 (6p21) [49]. This region encodes the major histocompatibility complex (MHC) I and II proteins that present antigens to T-cells. The locus contains about 250 genes, and about 40-60% of these have immune related functions. A characteristic of this region is the complicated linkage disequilibrium block structure. [50]. The HLA region comprises three subregions: the telomeric class I, class III, and the centromeric class II region, with class II as the major T1D risk region.

For T1D risk, the most important risk allele is HLA-DQB1 which is in linkage disequilibrium (LD) with HLA-DQA1 and HLA-DRB1 alleles and thus inherited as certain haplotypes. The DR4-DQ8 haplotype (*DRB1*0401/2/4/5-DQA1*0301-DQB1**0302) is the most common T1D predisposing haplotype in Finland, followed by *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) [51]. The HLA mediated T1D risk of an individual is determined by the two inherited haplotypes. The strongest risk genotype is the combination of the two risk haplotypes: DR3-DQ2/DR4-DQ8 and protection from T1D is dominant over risk alleles [52] (Table 1). By determining the HLA-DR-DQ haplotypes, the T1D risk of an individual can be estimated to be from 0,03% to 10%. Over 80% of all children developing T1D in Finland have a risk HLA genotype, whereas a protective genotype is present in 80% of healthy children [53]. Contribution of high-risk HLA genotypes on T1D development has been reported to decreased in time since individuals with lower HLA dependent risk profile are developing T1D [54].

Other alleles in the HLA region are also relevant to T1D risk. Of the HLA class I alleles, HLA-B seems to play a larger role than HLA-A or C [56]. HLA-B39, especially B*3906, conveys high risk. Other associated alleles are B7, B35, B44, and B57 [57]. It has been proposed that HLA class II genes are involved in the initiation of the autoimmune process, but other gene regions in its progression [55, 58]. For example, HLA-B*39 has been reported to enhance and A*03 protect against the progression of beta cell destruction after seroconversion [57]. Gene-gene or gene-environment interactions have also been described for HLA class I and II genes with age at onset [59-61]; the high risk DR3/DR4 has the highest frequency in the youngest patients, for instance.

Table 1. *Some of the common HLA class II haplotypes and genotypes conferring risk for T1D and some strong protective haplotypes and their odds rations in Finnish children. Modified from Ilonen et al. [55].*

Haplotypes/Genotypes	Odds ratio
Risk haplotypes	
DRB1*04:01-DQA1*03-DQB1*03:02	10.1
DRB1*04:05-DQA1*03-DQB1*03:02	3.0
DRB1*04:04-DQA1*03-DQB1*03:02	2.8
(DR3)-DQA1*05-DQB1*02	2.8
Protective haplotypes	
(DR7)-DQA1*02:01-DQB1*03:03	0.08
(DR15)-DQB1*06:01	0.07
(DR15)-DQB1*06:02	0.03
(DR14)-DQB1*05:03	0.03
Some common risk genotypes	
(DR3) - DQA1*05 - DQB1*02 / DRB1*0401 - DQA1*03 - DQB1*0302	14.7
(DR3) - DQA1*05 - DQB1*02 / DRB1*0404 - DQA1*03 - DQB1*0302	8.4
DRB1*0401 - DQA1*03 - DQB1*0302 / DRB1*0401 - DQA1*03 - DQB1*0302	7.6
(DR8) - DQB1*04 / DRB1*0401 - DQA1*03 - DQB1*0302	7.6
(DR1/10) - DQB1*0501 / DRB1*0401 - DQA1*03 - DQB1*0302	4.8
(DR3) - DQA1*05 - DQB1*02 / (DR3) - DQA1*05 - DQB1*02	4.1

Genetic risk loci outside the HLA region

In addition to HLA, more than 50 other gene loci to date have been confirmed to modify T1D risk [62] [In March 2017, ImmunoBase (www.immunobase.org) lists 57 loci associated with T1D]. Among the largest effect sizes described are insulin gene (*INS*, 11p15) and *PTPN22* (1p13) which were discovered early by candidate gene techniques together with *CTLA-4* (2q33) and *IL2RA* (Table 2). From 2006, genome wide association studies (GWAS) have multiplied the number of T1D risk genes. These studies started by GWAS of 4,253 cases and 5,842 controls [63], and soon evelvod to large studies combining different datasets; the largest to date included 9,934 cases and over 16,000 controls [64]. Recently, results on ImmunoChip analyses on T1D have been published [65]. ImmunoChip is a microarray involving over 200,000 SNPs/insertion-deletions associated with 11 immune-mediated diseases.

In general, if the HLA mediated risk for T1D is lower, the risk mediated by genes outside of the HLA region is relatively higher [66]. By genotyping these non-HLA genes in addition to HLA risk haplotypes, the development of future T1D or islet autoantibodies can be more reliably predicted [67-69].

In rare, monogenic diseases, the causal variant is usually a mutation in an exon of the causal gene leading to an altered amino acid composition and function. In complex, common diseases such as T1D, the associated genetic loci are usually common SNPs located outside gene bodies in regulatory regions. For many loci, the actual gene associating with the disease or how the mutation affects disease risk is unknown. Many are believed to exert their effects through gene expression (expression quantitative trait loci, eQTL) rather than affecting directly protein function. A SNP could for example influence the expression of a gene nearby rather than the gene it is situated in [70].

A limitation of GWAS is that the SNPs included are fairly common in the population (MAF>5%). This leaves out all the possible rare variants associated with T1D. Despite most being common SNPs, there are examples of rare loci associated with common disease. One example is IFIH1 in T1D risk; original discovery was a common SNP signal [71] which was later described being in LD with several rare variants [72].

The effect sizes of non-HLA T1D risk loci are small, and in total they explain a much smaller proportion of the heritablity of T1D than does HLA. Together all the risk loci discovered so far are estimated to explain 80-85% of the disease heritability [73]. Although this proportion is considerably higher than for other complex diseases, a part of the heritability is still unexplained. It has been proposed that still unknown non-HLA genes with small effect sizes or structural variants (insertions, deletions, duplications,

copy number variants, translocations and inversions) remain to be discovered. Additionally, epistatic interactions between these genes, leading to effects beyond the multiplicative or additive effect, might explain the missing heritability, although studies on T1D have not found substantial evidence for this [74, 75]. Importantly, not all familial clustering is explained by genes, but most environmental risk factors are also shared by family members.

The next paragraphs introduce some of the non-HLA risk loci.

Insulin gene (INS)

Insulin gene locus (11p15) has the strongest effect on T1D after HLA, and it was discovered already through linkage analysis [76]. Initially, the increased T1D risk was related to the variable number of tandem repeats (VNTR) region at 5' end of the insulin gene, with long repeats associated with T1D. Later, two polymorphisms (rs689) INS-23 A/T and +1140A/C have found in LD with the VNTR region [100], and might be in greater association with the causal variant than the VNTR region. The risk genotype increases T1D risk by lower insulin expression in thymus. This leads to low antigen presentation of insulin to the developing T cells and thus loss of central tolerance to insulin [101, 102].

The risk genotype of *INS* has been associated with the presence of IAA [20, 26], more precisely with the appearance of IAA as the first autoantibody [27]. An association has been described with the development of islet autoimmunity, but not with the progression of autoantibody positivity to clinical T1D [103, 104]. These associations were not observed in those with GADA as the first detectable antibody, but only in patients with IAA as the first antibody [104]. Thus, it is proposed that INS is involved in the initiation of the autoimmune process against insulin rather than the progression and expansion of this process [103]. This locus is not associated with other immune-mediated diseases.

Table 2. *Some key gene regions discovered to contribute to genetic predisposition to T1D.*

Year of publication	Number of new loci in the study:	Candidate loci/genes	Reference
1970s		*HLA class II*	
1984		*Insulin*	[76]
1990s		*HLA class I*	[77]
1996		*CTLA4*	[78, 79]
2004		*PTPN22*	[80]
2005		*IL2RA/CD25*	[81]
GWAS era:			
2006	1	*IFIH1*	[63]
2007	3	*12q13, 12q24 (C12orf30), 16p13 (CLEC16A)*	[82]
2007	4	*12q24, 12q13,16p13, 18p11 (PTPN2)*	[71]
2007	0	*CLEC16A*	[83]
2008	1	*12q13 (ERBB3)*	[84]
2008	5	*4q27 (IL2), 6q15 (BACH2), 10p15 (PRKCQ), 15q24 (CTSH), 22q13 (C1QTNF6)*	[85]
2008	6	*RGS1, IL18RAP, TAGAP, SH2B3, 3p21 (CCR5), PTPN2*	[86]
2008	1	*UBASH3A*	[87]
2009	1	*6q23 (TNFAIP3)*	[88]
2009	1	*12q13.3-q14.1 (KIF5A/CYP27B1)*	[89]
2009	18	*CD69/CLEC2D, GLIS3, IL10/PIK3C2B, etc.*	[66]
2009	1	*UBASH3A, BACH2*	[90]
2009	4	*four rare IFIH1 variants*	[72]
2009	2	*(FHOD3) 18q12, Xp22*	[91]
2010	2	*HERC2, IL26*	[92]
2011	3	*13q22, 2p23, 6q27*	[64]
2011	1	*FUT2*	[93]
2011	1	*AGER*	[94]
2011	1	*IKZF1*	[95]
2011	1	*SLC11A1*	[96]
2011	1	*LOC729653*	[97]
2014	9	*ITGB7, NRP1, BAD, CTSB, FYN, UBE2G1, MAP3K14, ITGB1, IL7R*	[98]
2015	7	*14q24.1, 17q21.31, 6q23.3, 1q32.1, 2q13, 4q32.3, 5p13.2*	[65]
2015	4	*1q24.3 (FASLG), 5q11.2 (ANKRD55), 6q23.3 (TNFAIP3), 7p12.2 (5´IKZF1 region)*	[99]

Protein tyrosine phosphatase, non-receptor type 22 (PTPN22)

PTPN22 is a strong risk gene, discovered already before the GWAS era [80]. It is located on 1p13 and encodes LYP, a protein tyrosine phosphatase. This polymorphism causes an aminoacid change (arginine to tryptophan) at amino acid position 620, which leads to gain of function with increased inhibition of T-cell receptor signaling. The risk allele alters T cell and, to some extent, B cell function but exact mechanisms leading to increased risk for T1D is not known. There are several hypotheses, however: First, poor T-cell receptor signaling in thymus could lead to poor negative selection of autoreactive T cells. Second, there are high levels of LYP-expressing dendritic cells, whose function might be altered. Third, poor function of regulatory T cells allows the expansion of autoreactive T-cells [105]. *PTPN22* has been associated with both the initiation of autoimmunity and progression from autoantibody positivity to clinical disease [103]. It predisposes also to multiple other AIDs [25].

Cytotoxic T lymphocyte antigen 4 (CTLA-4)

This gene is located on 2q31 and encodes CTLA-4, which is a downregulator of T cell function. When discovered, the susceptibility to T1D and autoimmune thyroiditis (AIT) was mapped to a commont allelic variant at a non-coding region of CTLA4, which was correlated with lower messenger RNA levels of the soluble alternative splice form of CTLA4 [78, 79]. This SNP is strongly correlated with thyroid autoimmunity [78, 79] and thus found on patients with both autoimmune thyroid disease and T1D [106].

Fucosyltransferase 2 (FUT2)

The FUT2 gene (19q13.4) codes for α(1,2)-fucosyltransferase, which synthesizes the H-antigen. This antigen is the precursor of the ABO blood group antigens on intestinal mucosa and body fluids. Being homozygous for the variant of nonfunctional protein (W143X rs601338A>G in Europeans), leads to nonsecretory status whereby no ABO-antigens are expressed in the intestine or saliva. The nonsecretors are resistant to norovirus infection, have a slower progression of HIV infection and protection from *Helicobacter pylori* infection. For these reasons, positive selection for the defected A allele has been suggested, as this is the major allele over the wild type G in many populations. On the other hand, the nonsecretors are more susceptible to *Streptococcus* infection, *Candida albicans* infection, Crohn's disease and T1D. FUT2 is a recessive T1D risk gene [93].

Interferon induced helicase C domain 1 (IFIH1)

IFIH1 codes for an intracellular pattern-recognition receptor which recognized double stranded viral RNA. It is involved in recognizion of picornaviruses. As enteroviruses belong to this family, the association might represent the link between virus infections and T1D pathology. The common nonsynonymous Ala946Thr variation on the gene (2q24) leads to T1D susceptibility [63] and later rare independent variants of the same gene have been discovered [72]. These variants, which lead to altered protein function, are associated with protection from T1D, leading to the suggestion that normal IFIH1 function increases T1D risk.

Epigenetics in type 1 diabetes

Epigenetics can be defined as processes that affect inheritance but do not depend on changes in DNA sequence. Examples of such processes are DNA methylation, gene silencing, inactivation of one of the X chromosomes in females, regulatory actions of noncoding RNAs (microRNAs, long noncoding RNAs), and making the DNA strand inaccessible for transcription factors by modifications on chromatin restructuring around histone structures [107]. In AIDs, epigenetic changes in either the effector immune cells or their target organs could affect the development of the disease. There are data indicating that epigenetic factors play a role for example in T helper cell differentiation, central tolerance induction, and autoantibody production by B cells. Especially the female preponderance seen in most AIDs could be mediated by epigenetics, perhaps by demethylation of the inactivated X chromosome in females [107]. In T1D, the histone methylation profile of T cells has been described aberrant for example in the promoter region of *CTLA4* [108], and T1D associated DNA methylation variable positions have been discovered from T1D discordant twins [109].

Environmental risk factors

Genetics alone can not explain the increase in T1D incidence over recent decades or the disconcordance between monozygotic twins. Accordingly, despite 70% of the general population carrying predisposing HLA genotypes, only 3-7% of those with a predisposing HLA genotype develop T1D. The incidence of T1D in children migrating to another region is at the level of the current region, not the region of origin [110].

Further, the incidence of T1D in Finland is six times higher compared to Russian Karelia despite similar genetic background [111]. These findings suggest the role of environmental factors in the etiology of T1D.

Environmental factors have been suggested to play a role in different stages of the disease process; some are believed to act as triggers in the initiation of the immune-mediated β-cell destruction, and some as promoters carrying this process forward. For example, in a German study, cesarean section associated with progression of islet autoimmunity to T1D, but not with the appearance of autoantibodies [112].

Despite a large range of hypotheses, T1D related environmental factors remain poorly defined. Most of the suggested environmental factors can be grouped under diet, infections, intestinal microbiota, toxins, or factors causing β-cell stress.

Diet

Different aspects of diet have been associated with T1D risk. Most of these relate to early infant feeding (e.g. breastfeeding, introduction of cow's milk or solid foods), but dietary factors later in life are also suggested (e.g. vitamin D or long-chain polyunsaturated fatty acid intake)

Breastfeeding as a protective factor for T1D development is still an open issue, although a meta-analysis showed limited protective effect [113]. Also breastfeeding during introduction of cereals in the infant's diet has been shown to protect from T1D [114]. Early exposure to cow's milk proteins has been proposed as a predisposing factor for T1D, but the recent studies have been contradictory. In Trial to Reduce IDDM in the Genetically at Risk (TRIGR), children with genetic risk for T1D received extensively hydrolysed infant formula, whenever breast milk was not available, as opposed to regular infant formula. In a smaller pilot study, weaning to extensively hydrolysed formula showed protective effect on development of islet autoimmunity [115], but the larger multinational TRIGR study did not confirm these findings by the first seven years of life [116]. Also increased intake of cow's milk later in life has been reported as a risk factor, but these findings have been contradicted as well [117, 118].

Both early (<4 months of life) and late (>6 months) introduction of solid foods, and especially gluten, in the infant's diet has been proposed a risk factor for islet autoimmunity [119, 120]. In a Finnish study, early exposure (<4 months) to root vegetables increased risk for islet autoimmunity [121].

Vitamin D deficiency has been proposed as a risk factor for T1D. The seasonal endogenous production of vitamin D by sun light could explain the lower rates of diagnosis of T1D in the summer. Also, lower or absent vitamin D supplementation has been implicated to increase the disease risk and higher serum 25-hydroxyvitamin D levels in pregnancy have been associated with decreased risk of T1D in the offspring. All these findings have been contradicted as well, and the question remains unanswered [11].

Administration of probiotics before the age of 28 days has been associated with decreased risk of islet autoimmunity. This finding was limited to children the high-risk HLA genotype DR3-DQ2/DR4-DQ8 and was absent among children with other genotypes [122]. Other nutritional factors associated with T1D risk are lower intake of long-chain polyunsaturated fatty acids and increased intake of nitrites, nitrates, or nitrosamines through food or water [11]. Also, advanced glycation end products and their receptor have been implicated in T1D development [123, 124].

Viral infections

Viruses have been implicated in T1D disease development, first due to the seasonal patterns of the two diseases coinciding in autumn and winter and later with serological and molecular methods for virus detection in patients. The most convincing evidence exists for enteroviruses (especially group B coxsackie viruses), although some reports for the involvement of other viruses, e.g. rotavirus, cytomegalovirus, and parvovirus, exists. Persistent infections of enteroviruses in the human pancreas and gut have been described in T1D. Enteroviruses along with some other viruses can cause diabetes in the NOD mouse model and these viruses infect and lyse cultured human islets in vitro [125].

The current hypothesis is that enteroviruses cause acute infection in the pancreatic islets leading to development of islet autoimmunity. In those individuals who fail to eradicate the virus, the infection continues in a slowly replicating chronic manner leading eventually to overt diabetes [126]. The hypothesis is supported by the notion that an excess in enterovirus infections is detected early in life already before seroconversion. Controversially, as T1D incidence has increased, incidence of enterovirus infections has decreased. To explain this phenomenon, so called polio hypothesis has been proposed; as the enterovirus exposure decreases, the immunity of newborns through maternal antibodies gets weaker and thus they are more susceptible to diabetogenic effects of enteroviruses. Vaccination agaist enteroviruses is an attractive candidate for primary

prevention of T1D and such intervention studies are also needed to determine the causality of enteroviruses in T1D [126].

Hygiene hypothesis

The hygiene hypothesis was first described for allergic diseases [127] and has later been extended to AIDs. The original hypothesis proposes that the reduced frequency of microbial infections in early life leads to increased risk to develop allergic or immune-mediated diseases. Evidence supporting the role of infections exists especially for atopic diseases, as e.g. measles, *Helicobacter pylori*, and *Toxoplasma gondii* infections are inversely associated with allergic diseases [128]. Not all studies support the hypothesis in its original form, however, as for example respiratory infections in early life (<6months of age) have been associated with increased risk of islet autoimmunity [11]. Accordingly, the hypothesis for association of microbial exposure and development of T1D now focuses more on commensal microbiota in the living environment rather than exposure to human pathogens i.e. the biodiversity hypothesis. To this end, the decreased biodiversity related to modern city living has been shown to alter human microbiota which affects the occurrence of allergic and inflammatory diseases [129]. A concrete example is that having an indoor dog in the family during the first year of life associates with protection from development of T1D related autoimmunity [130].

Microbiota

During recent years, microbiota has emerged as a new area of research due to development of analysis methods. The gut has a microbial flora with 100-fold more genes than the human genome, and we do not yet fully understand its functions. The colonization of the gut happens at birth – although some evidence exists for non-sterile environment in utero - and is mainly attributed to the microbial flora of the mother and family members. The microbiome develops through the first years of life and adult type microbial diversity is usually reached after the first three years of life. Mode of delivery (vaginal or cesarean) as well nutrition significantly affect the microbial flora. Several studies now indicate that lower gut microbial diversity is present in children with T1D related autoantibodies. Also, an abundance of butyrate- and lactate-producing bacteria has been described in children with islet autoantibodies. Considering these findings, it

seems that intestinal microbiota plays a role in progression of islet autoimmunity to clinical disease rather than initiation of autoimmunity [131]. It is still unclear whether this intestinal dysbiosis causes T1D development or whether it is a consequence of some other T1D related risk factor [132]. Recently, results of the DIABIMMUNE study have revealed marked differences in development of early intestinal microbiome in Finnish and Estonian infants compared to Russian Karelian infants. For example, *Bacteroides* species are dominant in Finland and Estonia, and the lipopolysaccharides (LPS) produced by these species (namely *B. dorei)* differ from those most abundant in Russians (namely *Escherichia coli* LPS). *B. dorei* LPS inhibits endotoxin tolerance and signaling of the innate immune system and and does not decrease incidence of autoimmune diabetes in NOD mice [133].

Factors inducing β-cell stress

Accelerator hypothesis proposes that insulin resistance related to higher weight gain or body mass index (BMI) results in the initiation of T1D related autoimmunity. The hypothesis has later evolved to implicate that a multitude of factors leading to increased insulin demand might have a role in development of T1D. Such factors are for example rapid growth, puberty, trauma, infections, overweight, and psychological stress [11].

Familial type 1 diabetes

Approximately 10-12% of children diagnosed with T1D have a first-degree relative with T1D at diagnosis, and if follow-up is continued for decades, this number increases to over 20% (Table 3). Compared to general population, first-degree relatives of T1D patients are at an 8-15-fold increased risk for T1D [134-140].
The proportion of children with T1D who have second-degree relatives with T1D has been reported to be 5-16% (Table 3). Compared to general population, the risk of T1D for second-degree relatives is approximately two-fold [134, 141]. Cumulative incidence of T1D for siblings of T1D patients is 3-6% by age of 20 years and the life time risk for parents is 2-6% (Table 4).

The proportions of familial T1D have not changed over time and the increase in incidence among first-degree relatives has been the same as for the whole general

population [137, 142]. A correlation exists, however, for the incidence of T1D in a population and the prevalence of familial disease [143]. Age at onset of T1D is higher in families with multiple children with T1D compared to families with only one affected child [144].

A peculiar sex-dependent inheritance pattern has been reported for T1D; fathers transmit the disease to their offspring more often than mothers [137, 160]. Appropriately, 4-7% of children have a father with T1D at diagnosis compared to 1.5-3% an affected mother (Table 3). Risk of T1D by the age of 20 years in offspring of fathers T1D was 7.8% and in offspring of mothers 5.3%, giving a 1.7-fold risk. This risk increases if the father was diagnosed with T1D in early childhood [137]. Additionally, there are reports on fathers transmitting T1D preferentially to daughters and mothers to sons [137, 138, 143], although not all studies have found this pattern [142, 160].

Reasons for the preferential transmission from diabetic fathers are still under debate. Many of the proposed mechanisms are genetic; greater haplotypic preservation of susceptibility gene loci in male patients (i.e. lower recombination frequency during gametogenesis), genetic susceptibility being preferentially transmitted from fathers regardless if they are diabetic or not, or genetic imprinting i.e. differential expression of the disease depending on the sex of the parent transmitting the susceptibility alleles [160, 161]. Evidence for these genetic differences is limited, however. For example, preferential transmission of T1D susceptibility from either parent has not been evident for HLA [162] or other loci; only one of 17 T1D loci showed some biased maternal transmission [163]. As risk of T1D development is similar in patients with fathers or siblings with T1D, and lower in patients with affected mothers, a protective effect of T1D in mothers has been suggested. Accordingly, a hypothesis of a protective influence on insulin secretion capacity of the fetus in diabetic pregnancies has been suggested. This hypothesis is supported by the epidemiological finding of a higher risk of T1D in children born before maternal diagnosis of T1D compared to children born after the diagnosis of the mother [164]. Additionally, maternal insulin treatment has been suggested to induce expansion of T regulatory cells (Tregs, CD4+CD25highFOXP3+ T cells) in the fetus: In a Finnish cohort of newborns, the proportion of circulating Tregs was higher in children with maternal T1D compared to children with unaffected mothers. In addition, insulin stimulation induced upregulation of molecules involved in activation of Tregs only in offspring of mothers with T1D [165].

Table 3. *Proportions of subjects with T1D who have relatives diagnosed with T1D.*

Reference	Country	number of subjects	age of subjects, years	% of subjects with 1. degree relative with T1D	% of subjects with sibling affected	% of subjects with parent affected	% of subjects with mother affected	% of subjects with father affected	% of subjects with 2. degree relative with T1D
At diagnosis of T1D:									
[141]	USA	194	<30 at dg	15.4	13.5	6.4	3.1	3.6	
[142]	Sweden	5824	<15 at dg	11.8	3.9	8.5	2.4	6.1	
[145]	Finland	801	8.4 at dg	11.2			2.6	5.6	
[143]	EuroDiab internation al -Finland	3960	<15 at dg	2.2-17.2 17.2	0.7-10.3 8.1		0-3.4 2.8	0-7.9 7.9	
[146]	Sweden	8538	<15 at dg		4	8.7	2.5	6.2	
[139]	Serbia	105	<16 at dg	4.8	2.9	1.9	0.9	0.9	16.2 (2. degree+cousins)
[147]	Ireland	283	<15 at dg	10.2	4.2	5.1	1.4	3.7	16.7
After follow-up:									
[144]	USA	1128	<17 at dg, mean duration 6.8	11.1	6.7	5	1.4	3.7	
[148]	Denmark	1419	8.6 at dg	12.8	5		2.1	6.7	
[149]	Denmark	291	22 at dg, median duration 40	25.1 (after 40 yrs of T1D), 10.4 at age 21	16.5		2.4	3.8	
[138]	Finland	5255 parents, 9453 offspring	<30 at dg, follow-up 7.7	15.1			2.6	5.9	
[150]	UK	1641	<21 at dg			8.6			
[151]	UK	505 families	24.2			6.2	2.9	9.5	
[152]	Sweden	2537	15-34 at dg	16.5	8.3	9.6	4.2	5.8	
[153]	USA	1586	<16 at dg, median duration 3.5	8.1					
[134]	USA	2691	all ages						4.8
[154]	Brazil	145	17.3 at dg, mean duration 13.1	13.8					6.2 (2. and 3. dgr relatives)
[155]	Finland	300	6.7 at dg, 11.9 at the time of study	15	8.3	7	2	5	16.3
[156]	Italy	461	<15	7					5.1

Table 4. *Risk of being diagnosed with T1D for relatives of T1D patients.*

Reference	Country	Total n	Risk of siblings	Risk of offsping	Risk of parents
[144]	USA	1128	3.3% by 20 yrs		
[149]	Denmark	291	9.6% by 60 yrs	6.3% by 34 yrs	
[157]	USA	453 T1D fathers, 696 T1D mothers		by 20 yrs 5.4% offspring of fathers, 2.1% for offspring of mothers	
[138]	Finland	5255 parents, 9453 offspring		5.3% by 20 yrs; fathers 7.6%, mothers 3.5%	
[150]	UK	3164			5.7% by 60 yrs
[158]	UK	1299	4.3% by 30 yrs (11.7% if proband dg<5 yrs)		4.2% by 40 yrs, fathers: 5.7%, mothers 2.8%
[159]	Finland	5144 index cases, 10 168 siblings of T1D children	6.9% by 50 yrs		
[153]	USA	1586	4.4% by 20 yrs (6.9% if proband dg<7 yrs)		2.6% by 40 yrs, fathers 3.6%, mothers 1.7%
[137]	Finland	5144 index cases, 5291 offspring		6.7% by 20 yrs	
[141]		194	9% by 20 yrs		

An additional explanation for the lower risk for T1D in children with mothers with T1D would be selective spontaneus abortion of pregnancies with fetuses having high genetic risk for T1D. In fact, risk for miscarriage is higher in women with diabetes compared to healthy women and this risk increases if diabetes was diagnosed at an early age. This has been explained by hyperglycemic state during fertilization and early pregnancy [137]. Fertility is reduced in women with T1D, but also in men with T1D, albeit to a smaller degree [166, 167]. Older maternal age at birth has been reported as a protective factor for T1D, a finding independent of birth order, maternal age at first pregnancy, interval between pregnancies or diabetes diagnosis before or after pregnancy [164].

Familial clustering of T1D is likely caused by both genetic and environmental risk factors. The risk-associated HLA genotypes have been observed more often in familial T1D [145, 168-170] although not all studies have found significant differences [171, 172]. It has been proposed that familial T1D is mainly attributed to HLA class II genes and not genes outside the HLA region [74]. Support comes from GWA studies where the effect sizes for non-HLA loci were smaller in families with affected siblings than in sporadic cases [66].

Two studies have noticed no differences in diabetes-associated autoantibodies, e.g. insulin antibodies (IAA), glutamic acid decarboxylase antibodies (GADA) [145], or islet cell antibodies (ICA) [145, 168] suggesting similar pathogenetic mechanisms in familial and sporadic forms of the disease. A study from Israel reported, however, higher frequencies of IAA as well as a higher number of positive antibody responses among familial cases [173]. The development from autoantibody positivity to clinical T1D seemed similar in a large prospective report for cohorts of familial T1D and sporadic cases recruited from the general population [16].

Clinical status of the child at diagnosis is less severe in families with prior experience of the disease in a first-degree family member [145, 173, 174]. This can be easily explained by the better awareness and alertness of the family and the family physician of the early symptoms of T1D. Consequently, T1D is diagnosed earlier leading to milder onset and for example lower prevalence of diabetic ketoacidosis. During follow-up after T1D diagnosis, children with familial diabetes had higher long-term HbA1c in Denmark [175].

Familial T1D has been associated with younger age at onset of T1D although not by all studies. An Italian study reported earlier age at onset in children with family history of T1D in second-degree relatives compared to sporadic cases (5 vs. 8 years, P=0.001) [156].

Associations with general autoimmunity

Patients with T1D are at an increased risk for other AIDs, for example CD and autoimmune thyroid disease [176]. Relatives are at an increased risk for T1D and other AIDs as well. This clustering of diseases could be attributed to genetic and environmental factors predisposing not just to one AID but to autoimmunity in general.

Epidemiology of additional autoimmunity

T1D is associated with other autoimmune/immune-mediated disorders such as autoimmune thyroiditis (AIT), celiac disease (CD), Addison's disease, pernicious anemia, rheumatoid arthritis, and multiple sclerosis (MS). AIT is the most common comorbid condition with a prevalence of 15-30%. CD has been reported in 4-9%, Addison's disease in 0.5%, autoimmune gastritis in 5-10%, and vitiligo in 2-10% of patients with T1D [177]. Increased risk of multiple autoimmune manifestations exists already at diagnosis of T1D but increases significantly with follow-up [178, 179]. After screening for autoantibodies at diagnosis, 6% of children with T1D have been reported to have another AID [180]. Celiac disease (1.5-3.3%) [180-183] and AIT (0.6-3.1%) [33, 180, 183] are the most common conditions already present at T1D diagnosis. For example, older age and female gender are risk factors for developing additional autoimmunity in T1D patients (Table 5).

The risk for AIDs is increased also in the relatives of T1D patients. In a recent meta-analysis, first-degree relatives of T1D patients were at significantly increased risk for Addison's disease, AIT, ancylosing spondylitis, CD, MS, pernicious anemia, primary biliary cirrhosis, psoriasis, RA, SLE, vitiligo, and Wegener's granulomatosis [188]. AIT, CD and rheumatoid arthritis are the most common conditions [151, 178, 189, 190]. The risk for the relatives is even greater in families where the index child with T1D has multiple AIDs [189], and the risk for an additional AID is higher if the patient is already diagnosed with an AID [151].

Table 5. *Some reported risk factors for development of additional autoimmune diseases in T1D.*

Risk factors for developing additional autoimmune diseases (AID) in addition to type 1 diabetes (T1D)	**References**
Female gender	[34, 178, 179, 184]
Long duration of T1D	[34]
Older age at onset of T1D	[34]
Older age	[34, 184]
Positive family history of AID	[34]
Positivity and high titers of GADA	[34]
Negativity or low levels of IA-2A	[34]
Risk factors for developing celiac disease (CD):	
Female gender	[34, 185]
Long duration of T1D	[34]
Young age at onset of T1D	[34, 182, 185]
First-degree relative with an AID	[34, 185]
IA-2A negativity	[34]
HLA-DR3-DQ2	[34, 186, 187]

Due to the co-occurrence of AIDs, screening of patients with T1D has been recommended. In Finland, anti-tTG and thyroid function tests are performed at diagnosis and at regular intervals (every one or two years) in children diagnosed with T1D. Other markers of AIDs are measured if symptoms occur or suspicion is otherwise raised. These protocols may differ locally, however. Different recommendations have been suggested by international organizations (Table 6). Additionally, regular measurements of ACA and PCA have been suggested by some [191, 192].

Table 6. *Recommended screening protocols for associated AIDs in patients with T1D.*

Recommending institution	Thyroid autoimmunity	Celiac autoimmunity	Adrenal autoimmunity	Reference
ISPAD (International Society for Pediatric and Adolescent Diabetes)	TSH and TPO-Ab at diagnosis and every second year thereafter in TPO negative children without symptoms or goiter	anti-tTG and/or EMA at diagnosis and every 1-2 years thereafter, more frequently if symptoms or first-degree relatives with CD	if symptomatic or otherwise suspected	[193]
American Diabetes Association (ADA)	TPO-Ab and anti-tTG soon after diagnosis, TSH after diagnosis when glucose control has been established and every 1-2 years thereafter	anti-tTG or deamidated gliadin antibodies soon after diagnosis and 2 and 5 years thereafter or if symtomatic or first-degree relatives with CD	assessed and monitored as clinically indicated	[194]
Current Care Guidelines (Käypä Hoito), Finland (for patients >16 years of age)	TSH at diagnosis (antibodies if TSH abnormal), TSH every 5 years	celiac autoantibodies at diagnosis and every 5 years until age 20		[195]

Thyroid autoimmunity

Clinical autoimmune thyroiditis (AIT, Hashimoto's thyroiditis and Graves' disease) is the most common AID associated with T1D. Hashimoto's thyroiditis (manifesting mostly as hypothyroidism) is more common than Graves' disease (hyperthyroidism). Thyroid autoimmunity is manifested as autoantibodies against thyroglobulin (Tg-Ab), thyreoperoxidase (TPO-Ab) and TSH receptor. These antibodies are present in 12-23% of pediatric patients with T1D and in 20-40% of adult patients with T1D [192]. The prevalence is higher among girls, and increases with increasing age and duration of T1D. Of those T1D patients with positive thyroid autoantibodies, 6-72% also have clinical or subclinical thyroid dysfunction, whereas of the healthy controls with these autoantibodies, only 0-25% [191]. Clinical hypothyroidism is diagnosed in 4-18% of T1D patients [192].

Thyroid autoimmunity usually develops a few years after the T1D diagnosis and only very rarely precedes it. Diabetic ketoacidosis and disturbances in glycemic control affect thyroid function and can cause a great number of false positive findings if thyroid function is assessed at diagnosis: 19% of pediatric patients had thyroid hormone disturbances at diagnosis and only 2.7% required treatment after two years of follow-up [196].

Celiac disease (CD)

The second most common co-occurring AID in T1D is CD; biopsy-proven CD in pediatric patients with T1D is diagnosed in 4-6% [197-199]. In CD, intestinal mucosa is destroyed by the immune system in response to gluten containing cereals (wheat, rye, barley). Treatment is life-long gluten-free diet. Celiac autoimmunity is usually evident by detectable autoantibodies: tissue transglutaminase autoantibodies (anti-tTG), endomysial antibodies (EMA), and autoantibodies against deamidated gliadin peptides (DGP) [200]. CD is discussed in detail later in this section.

Adrenal autoimmunity

Autoimmune reaction targeted against adrenal tissue results in adrenal insufficiency (Addison's disease, AD) with impaired production of glucorticoids, mineralocorticoids and androgens. Adrenocortical autoantibodies (ACA) are detected in 0-4% of patients with T1D compared to 0-0.7% of controls [191]. These autoantibodies recognize the adrenal 21-hydroxylase, a P450-cytochromal enzyme. In Finnish pediatric population, 2.3% of T1D patients developed ACA during the follow-up of 3-4 years, but none developed clinical adrenal dysfunction [201]. Usually, clinical disease develops in 3.3-

40% of T1D patients with ACA [191]. The prevalence of co-occurring AD and T1D (Schmidt syndrome) in adult patients is 0.8-1.9% [192].

Autoimmune gastritis

Autoimmune gastritis is an autoimmune reaction against the gastric H+/K+ ATPase enzyme in gastric parietal cells. The disease starts as a symptomless gastritis and leads to atrophic gastritis and eventually pernicious anemia (vitamin B12 and iron deficiency). The process is manifested as autoantibodies against gastric parietal cells (parietal cell antibodies, PCA). The prevalence of PCA among T1D patients is 3-34% (5-8% in pediatric patients), and among the general population 0-13% [191, 192]. Atrophic gastritis develops in 43-50% of T1D patients positive for PCA, but pernicious anemia develops only in 1-23% [191]. Overt gastritis in pediatric T1D patiens is rare.

Other autoimmune diseases

Vitiligo is manifested as white spots on the skin due to loss of epidermal melanocytes. It is estimated to be 10-20 times more frequent in T1D patients than in general population with prevalence in pediatric T1D patients reported to be 6% [192].

T1D and MS cluster together despite their conflicting HLA predisposition; HLA-DRB1*1501-DQA1*0102-B1*0602 predisposes to MS but protects from T1D [202]. In a Danish cohort, patients with T1D were at a three-fold increased risk for MS [135].

Juvenile idiopathic arthritis is associated with T1D; in a multicenter study the prevalence was 0.2% in pediatric T1D patients compared to 0.05% in general population [203].

Autoimmune polyglandular syndromes (APS) are characterized by the presense of multiple AIDs in an individual. APS-1, also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is a monogenic, autosomal recessive condition caused by a mutation in autoimmune regulator gene (AIRE). These patients develop adrenal insufficiency, hypoparathyroidism, and mucocutaneous candidiasis usually already during childhood. Other associated conditions are T1D, vitiligo, alopecia, hepatitis, pernicious anemia, primary hypothyroidism, hypergonadotropic hypogonadism, and malabsorption. APS-2 is more common in adults and manifests as adrenal insufficiency and other AIDs including T1D. APS-3 involves multiple endocrine gland AIDs but usually not adrenal insufficiency. APS-2 and APS-3 are polygenic conditions associated with HLA class II haplotypes [192, 193].

Atopic disorders

Traditionally, autoimmune and atopic diseases are thought to arise from different pathways of immune reactions and thus rarely coexists. Atopic diseases are driven by Th2-type immune response whereas Th1-type in dominated in AIDs. As these two are mutually inhibitory, in theory the two types of diseases should not coexists. There are data to support this view: a meta-analysis found a reduction in prevalence in asthma in T1D patiens, although for other atopic diseases the results were inconclusive [204]. In Danish twins, the risk for atopic dermatitis was clearly decreased in patiens with T1D [205]. There are, however, recent data that place these two disease modalities closer together [50, 206]. Asthma prevalence among children and adolescents with T1D in the USA was 10.0% whereas the estimate for general population was 8.7% [207]. The incidences of T1D and asthma/atopic eczema correlate at country-level, both increasing in developed countries [208]. This points to mutual predisposing environmental risk factors, with reduced microbial exposure as the most prominent [209]. When studying the Th1/Th2 cytokine profiles of patients with both T1D and asthma, it has in fact been discovered that these patients have a unique secretory pattern, which combines elements from both diseases [210-212]. These diseases also share predisposing genetic factors [213].

Etiology and pathogenesis of additional autoimmunity

Risk factors for many AIDs are shared. For example, almost all AIDs are more prevalent in women/girls. T1D is a striking exception to this rule; in children under the age of 15, there is a male excess in countries with high T1D incidence whereas many low incidence countries report female excess. After puberty, however, populations of European origin report a clear male excess in T1D [214]. Norwegian data suggests that similar male excess in T1D has been evident since the beginning of the 20th century [214]. In MS, however, the risk for the disease is higher in female patients, but this situation has developed relatively recently as the sex ratio used to be equal some 50 years ago [215].

Similar environmental risk factors have been associated with many AIDs. For example, enterovirus infections have been suggested in T1D and Epstein-Barr virus (EBV) in RA, MS, and Sjögren's syndrome [216]. The gastrointestinal tract has been implicated as a site of initiation of autoimmunity in multiple AIDs. Accordingly, nutritional factors such as duration of breast feeding, vitamin D deficiency, or timing of introduction of solid foods such as cow's milk or gluten containing cereals have been studied in many AIDs.

This has led to similar attempts at primary prevention of different immune mediated diseases. For example, extensively hydrolyzed formula has been studied in allergic diseases in addition to T1D. In a German study, children with parental history of atopy received hydrolysed formula as breast milk substitute if necessary for the first four months of life. A reduction of risk of allergic dermatitis was evident and the effect has now lasted up to the age of 15 [217]. A recent meta-analysis on hydrolysed formula and risk of allergic diseases does not support these claims, however [218]. Moreover, a meta-analysis from the same group concluded that no evidence exists for timing of allergenic food introduction and development of CD, T1D or other AIDs. Reduction of egg allergy and peanut allergy, however, were reported for egg introduction at 4 to 6 months and peanut introduction at 4 to 11 months [219].

Hygiene hypothesis, i.e. the decreased microbial exposure during childhood increasing the incidence of atopic diseases, has been extended from allergic diseases to development of autoimmunity. Accordingly, sharp contrast exists between prevalences of immune-mediated diseases in Finland and Russian Karelia; the incidencies of T1D and CD, as well as prevalences of thyroid autoantibodies and allergen specific IgE were 3-6 times higher in Finland [128]. These two geographical areas are neighbourgs with limited differences in HLA genetics but marked differences in living conditions and socioeconomic status. For example, many infections such as Helicobacter pylori, hepatitis A, and Toxoplasma gondii are still common in Russian Karelia but infrequent in Finland. Also, the microbial composition of household dust differs in these areas in accordance with the hypothesis of decreased biodiversity increasing the risk of immune-mediated diseases [128]. Accordingly, contact with farm animals, indoor dogs or cats, having multiple siblings, or attending day care early have been implicated in protection from many allergic and autoimmune diseases [128].

These results highlight that, despite the multiple phenotypes of autoimmunity, many proposed etiological factors are strikingly similar. This is understandable as the reactivity of the immune system and loss of tolerance to self-antigens are proposed mechanism behind all. The phenotypes of these AIDs are very different, however. Factors determining the target tissues for these specific autoimmune phenotypes are unknown. Some unique genes have been described, e.g. *INS* is a risk locus only for T1D and *TSHR* (encoding TSH receptor) only for AIT.

Genetics of autoimmunity

HLA region

HLA region is associated with all AIDs. The haplotypes DR4-DQ8 and DR3-DQ2 are the major contributors to genetic risk of T1D among Caucasians. Both of these, but especially DR3-DQ2, have been associated also with other AIDs. CD and T1D share the same HLA background, as 90% of patients with CD have DR3-DQ2 [220] and an overwhelming majority of the rest DR4-DQ8 haplotype. DR3-DQ2 has been associated also with other conditions, such as SLE, myasthenia gravis, idiopathic inflammatory myositis, stiff person syndrome, and Addison's disease [221]. Addison's disease has been associated with the rare DR3-DQ2/DRB1*0404-DQ8 genotype; 30% of patients carry this genotype compared to less than 1% of the general population [222]. Different DRB1*04 alleles are associated with rheumatoid arthritis, autoimmune hepatitis, alopecia, juvenile idiopathic arthritis, and Crohn's disease [221].

Genes outside the HLA region

Most genes associated with AIDs are shared with other AIDs [50, 223]. In a recent review, 127 non-HLA loci were associated with the six common AIDs (T1D, rheumatoid arthritis, CD, AIT, psoriasis, and SLE). More than half of these SNPs were shared with other AIDs, for example CD and RA as well as CD and T1D share over 50% of the associated loci [223]. To make matters more complicated, the risk allele is not always the same between diseases. For example, for the *PTPN22* SNP rs2476601, the A allele yields susceptibility to T1D and RA [66] and G allele for Crohn's disease [92]. *IL10, IL27*, and *IL18RAP* also have opposite direction of effect for T1D or IBD [92], and *IL18RAP* opposite effect for CD [86]. Accordingly, it has been suggested that, genetically, T1D is closest to other AIDs with autoantibodies, particularly juvenile idiopathic arthritis, whereas T1D is least similar to ulcerative colitis [65].

The genes associated with AIDs can be grouped under three major pathways; genes involved in T cell differentiation; immune cell activation and signaling; and innate immunity and TNF signaling. Of the genes not included in these pathways, the genes associated with inflammatory bowel diseases (Crohn's disease and ulcerative colitis) form a distict group, as do genes associated with other cytokines and chemokines [50].

Most AIDs have susceptibility genes involved in all main three pathways highlighting the common overlap of pathogenesis. Despite extensive studies, only 50% of the assumed heritability of autoimmunity has been explained [223].

Celiac disease

Celiac disease (CD) is an immune-mediated disease evoked by dietary gluten and related prolamins and characterized by autoantibodies, predisposing HLA genotypes, and enteropathy. The HLA predisposition (DQ2 and DQ8) is shared with T1D, and accordingly patients with one disease and their relatives are at an increased risk for the other [189, 224]. Other risk groups for development of CD are for example patients with Down syndrome, AIT, Turner syndrome, IgA defieciency, and first-degree relatives of CD patients [200]. CD is treated with a life-long gluten free diet, which allows the intestinal mucosa to heal, alleviates symptoms and results in disappearance of autoantibodies.

The clinical symptoms and signs of CD are variable and may include systemic manifestations in addition to enteropathy. Classic CD symptoms and signs in children include diarrhea, weight loss, failure to thrive, and abdominal pain. Additionally, CD can cause iron deficiency anemia, aphthous stomatitis, and dermatitis herpetiformis rash. However, increasing proportion of patients are asymptomatic at the time of diagnosis [200].

Tissue transglutaminase type 2 (tTG) is the primary autoantigen in CD [225], and accordingly tissue transglutaminase autoantibodies (anti-tTG) are used for the screening of CD autoimmunity. Endomysial antibodies (EMA) are detected by immunofluorescent methods and are the most specific method for detection of CD autoimmunity. Also antibodies against deamidated gliadin peptides (GDP) are often detectable especially among very young children with CD. Autoantibodies against native gliadin (anti-gliadin antibodies, AGA) are not sensitive or specific enough for the detection of CD [200].

CD related enteropathy is characterized by a wide range of changes from increased infiltration of the mucosa by the cells of the immune system to partial or total villous atrophy. Marsh classification is used to grade the severity of the observed enteropathy (0 – no villous atrophy, 3 – villous atrophy). Accordingly, histological evaluation of intestinal biopsy samples is the golden standard for CD diagnosis. However, The European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)

guidelines from 2012 recommend that intestinal biopsy may be omitted in symptomatic children with predisposing HLA haplotypes and high IgA-anti-tTG levels (>10 times of the upper limit of normal) and EMA [200].

Epidemiology of celiac disease in T1D

T1D patients are at 5-7-fold increased risk of CD [198]. Compared to approximately 1% in the general population [226, 227], the prevalence of biopsy-proven CD in pediatric patients with T1D is 4-6% [197-199]. The variation is large, however, ranging from Finland's 2.4% to Algeria's 16.4% (Table 7). CD is mostly diagnosed after the T1D diagnosis. Accordingly, only 0.5% report CD before T1D diagnosis [181, 182, 197, 228, 229]. At T1D diagnosis, 3.9% to 16.8% of patients are positive for anti-tTG (Table 7).

Most patients with CD are asymptomatic at diagnosis, i.e. are found by screening. In the US, only 40% of new pediatric CD cases and 59% of adult cases were symptomatic [250]. Most of them do feel better after initiation of gluten free diet, however. Among T1D patients, 74% of those diagnosed with CD were asymptomatic [238]. Risk of silent CD was especially high among first-degree relatives of CD patients who themselves had an AID diagnosis compared to those without such a diagnosis (25% vs 4.7%). This emphasizes that CD in combination with other AIDs is often asymptomatic and screening of risk populations has been recommended [200].

The data on whether or not undiagnosed CD has adverse outcomes on glycemic control of T1D or on growth of the child are conflicting [238]. Untreated CD patients have an increased risk for iron deficiency anemia, growth retardation, osteoporosis, neuropsychiatric disorders, fertility problems, and intestinal lymphoma. Although data on long-term risk or benefit of gluten free diet on asymptomatic children diagnosed with CD by routine screening is scarce, ESPGHAN recommendation is to start gluten free diet [200].

Table 7. *Frequency of CD and celiac autoimmunity in patients with T1D.*

Study population	Country	At diagnosis of T1D, %	During follow-up, %	Reference
Celiac disease				
pediatric	Israel		8.3	[189]
all ages	Belgium		2.3	[230]
1-27 yrs	Germany		1.7	[231]
pediatric	Australia	2.4		[183]
pediatric	Finland		4.7	[155]
pediatric	USA	2.9		[180]
pediatric	Greece		3.5	[232]
pediatric	Greece		4.8	[233]
all ages	international		2.7	[34]
pediatric	USA		2.3	[234]
pediatric	USA		1.8	[235]
pediatric	Finland		2.1	[236]
pediatric	Sweden	3.3	10	[182]
pediatric	Finland	1.2	2.4	[181]
pediatric	Italy	3.6	6.2	[228]
0.8-22.4 yrs	UK		0.5	[237]
5-35 yrs	Israel	0.1-1.3	5.5	[238]
pediatric	Algeria		16.4	[239]
all ages	Italy	5.0		[240]
pediatric	Italy		7.6	[185]
not pediatric	Italy	0.6	5.7	[241]
pediatric	Israel		4.0	[242]
pediatric	Sweden		6.7	[229]
pediatric	Australia		4.2	[243]
pediatric	Estonia		3.3	[244]
Anti-tTG				
1-27 yrs	Germany		4.4	[231]
pediatric	USA		11.6	[235]
<30 yrs	Germany/Austria		10.7	[245]
pediatric	USA	11.6		[180]
pediatric	Greece		7.6	[232]
pediatric	Greece	5.7	8.6	[233]
pediatric	USA		10.1	[234]
partly pediatric	Germany	3.9		[246]
mostly pediatric	Italy	8		[247]
mostly pediatric	Germany	16.8		[248]
0.8-22.4 yrs	UK	25	13.4	[237]
partly pediatric	Germany	10.1		[249]
pediatric	Italy		15.1	[240]
pediatric	Israel		6.5	[242]
EMA				
pediatric	Australia	2.4		[183]
pediatric	Italy	5.5	9.9	[228]
all ages	USA		4.4	[250]
all ages	Italy		5.7	[241]

Pathogenesis and etiology of celiac disease

The strongest CD risk haplotype is HLA-DR3-DQ2. This haplotype is evident in approximately 90% of children with CD and 55% of children with T1D [220], compared to 20 to 25% of the general population. Somewhat lower figures have been reported in Finland, suggested to partly explain the lower prevalences of CD in patients with T1D [236]. The DR3-DQ2 haplotype exerts CD risk in an additive manner, with homozygotes at highest risk [251].

Not everyone with the susceptible HLA haplotype develop CD, however, which highlights the importance of environmental factors. The timing of introduction of gluten and breast feeding during this introduction have been suggested to be related to CD risk. The epidemic of CD in Sweden during the 80's coincided with greater gluten consumption after 6 months of age [252]. Recent randomized, controlled trials have not confirmed these findigs, however. A European multicenter study (PreventCD) [253] and an Italian study [254] detected no effect of timing to gluten introduction (4-6months or 6 months vs. 12 months of age). Breast feeding, neither exclusive nor during gluten introduction, influenced later development of CD. Other environmental risk factors related to CD are elective cesarean section, perinatal and childhood infections, and the use of antibiotics and proton-pump inhibitors [255, 256].

Female gender is a risk a factor for CD [251], although not evident in all populations [250]. For CD in T1D, reports both for and agaist increased risk in female patients exists. In Italy and Estonia, more girls have been reported with the double diagnosis [240, 244, 257, 258], whereas in Sweden and Autralia both genders have been affected equally [182, 229, 243]. Additionally, young age at diagnosis of T1D, first-degree relatives with AIDs, IA-2A negativity, and HLA-DR3-DQ2 have been associated with risk of CD in patients with T1D (Table 5).

Comorbid AIDs related to CD are usually diagnosed before the diagnosis of CD. In T1D, most CD cases are diagnosed within two years after T1D diagnosis. It has been speculated that the metabolic derangement at diagnosis of T1D could accelerate the autoimmune process related to CD and thus lead to increased incidence of CD after T1D diagnosis [182]. Also, it has been hypothesized that continuing gluten exposure in silent CD might increase the risk for other AIDs [259].

Heterogeneity within T1D

As knowledge of the T1D disease process has accumulated, we have come to understand that T1D is characterized by considerable heterogeneity. The genetic etiology, timing of autoantibody appearance, large range in age at onset of the disease, and the highly variable length of the prediabetic process highlight the variability seen in the disease. This heterogeneity could reflect various pathogenetic mechanisms of T1D. In fact, differential disease processes have been proposed for example depending on the first autoantibody to appear during the prediabetic disease process: IAA as the first autoantibody associates with *INS* risk genotype, *PTPN22* risk genotype, HLA-DR4-DQ8, and early age at seroconversion. In contrast, GADA as the primary autoantigen develops later and in children with HLA-DR3-DQ2 [25-27]. In Finnish data, *IKZF4-ERBB3* was associated with the progression of T1D after development of islet autoimmunity only in subjects with GADA as their first antibody, whereas *INS* associated with seroconversion only in children with IAA as the first autoantibody [104]. These pathogenetic pathways with different first autoantibodies could possibly operate together with a synergistic effect. This hypothesis is supported with the enhanced genetic risk associated with HLA-DR3-DQ2/DR4-DQ8 heterozygosity.

The different pathogenetic pathways could possibly be initiated or promoted through different etiological triggers. In fact, such gene-environment interactions have been described. For example, the *PTPN22* gene polymorphism affects the rate of diabetes-associated autoimmunity and clinical disease only in children who were introduced to cow´s milk based infant formula before the age of six months [260]. Cesarean section increases T1D risk only in children with high risk *IFIH1* genotypes [112]. Examples of gene-gene interactions include the increased risk effect of *PTPN22* in those negative for DR3-DQ2/DR4-DQ8 genotype [25, 75, 261] and a stronger protective effect of *CTLA-4* in those with DR4-DQ8/nonDR3-DQ2 or DR3-DQ2/nonDR4-DQ8 [75].

Heterogeneity in genetics has been described according to age at onset. Risk alleles in *IL2* and *RNLS* genes have been associated with younger age at onset [75] and rs10517086 has been associated with islet autoimmunity in those children under the age of 2 years [262]. Also, disease variants characterized by the rate of progression to T1D have been described. In a German study, the children progressing rapidly (<3years) from islet autoimmunity to clinical T1D were distinguished from slow progressors by an overall higher genetic load of alleles predisposing to T1D. The development of IA-2A in slow progressors was delayed, and rapid progressors were delivered by cesarean section [41].

Different pathogenetic mechanisms of T1D have also been suggested in isolated T1D and in T1D associated with a broader autoimmune activation reflected by multiple AIDs [230, 263]. The double diagnosis of T1D and CD has been associated with two non-HLA loci, viz. *CTLA4* and *IL2RA* [187].

This kind of subtyping of the disease helps us to understand the pathogenetic mechanisms and natural course of the disease, eventually making it possible to identify means for prediction and prevention of the disease, which might depend on the disease subtype.

Aims of the study

This thesis is focused on determining the frequency of additional autoimmunity in Finnish children newly diagnosed with T1D and in their extended family members, and characterizing the effects of this additional autoimmunity on clinical, metabolic, and genetic markers as well as T1D related autoantibodies.

The specific aims of this thesis were:

1. To collect current data for frequencies of additional AIDs among Finnish children under the age of 15 with newly diagnosed T1D and the frequencies of T1D and other AIDs in their extended family.

2. To compare children with familial and sporadic T1D in terms of T1D related autoantibodies as well as clinical, metabolic, and HLA class II genetic markers: A stronger genetic susceptibility to T1D and a milder metabolic decompensation in children with a positive family history for T1D was expected, whereas no differences were predicted in the autoantibody profile.

3. To compare children with personal or family history of AIDs other than T1D in terms of T1D related autoantibodies as well as clinical, metabolic, and HLA class II genetic markers: These children were expected to have an increased prevalence of DR3-DQ2 and a stronger autoimmune reactivity against beta cell antigens manifested as more frequent autoantibodies with higher titers. The rationale being that, if the immune system is broadly active as evident by multiple AIDs in the patient or their family members, it could be assumed to be broadly active also against autoantigens of one disease.

4. To characterize T1D co-occurring with CD or CD related autoimmunity: overrepresentation of HLA-DR3-DQ2 positives and a broader β-cell-specific autoimmunity were expected.

5. To evaluate the effect of non-HLA gene loci on clustering of autoimmunity in individuals and families with T1D.

Subjects and methods

Study subjects

All the subjects of this thesis were participants of the Finnish Pediatric Diabetes Register and Sample Repository. This is a nation-wide project initiated in 2002 to study childhood diabetes in Finland. All children diagnosed with diabetes in Finland and their families are invited to participate by the hospitals handling the T1D diagnosis. The Register includes a questionnaire (Supplement of Study I) with questions on the family structure, diseases of the child and family members, as well as clinical markers at diagnosis of the child (blood glucose, pH, duration of diabetes symptoms, etc.). The index children and their first-degree relatives (mothers, fathers, siblings) are asked to give blood samples to the Sample Repository. The samples are analyzed for islet autoantibodies (ICA, IAA, GADA, IA-2A, ZnT8) and HLA class II DR- and DQ-haplotypes. The T1D risk mediated by these haplotypes is classified into risk classes. The Register does not include systematic follow-up of the cases, but if an additional member of the family is diagnosed with diabetes in a participating pediatric center and registered, the family information is updated accordingly.

In 2009, the coverage of the Register was investigated over a three-year period by asking pediatric hospitals to record all the patients diagnosed at their center and comparing this information on that found in the Register. The proportion of newly diagnosed children registered was 92% [264]. Approximately 70% of those registered give samples for the Sample Repository.

Study I and II

For studies I (Familial T1D) and II (Other AIDs), data on 1488 children were collected from the Register. The median age at diagnosis of T1D was 8.2 years, 56.9% of the subjects were male. At data collection in October 2006, the Register included 2662 children with T1D. Children diagnosed at 15 years of age or later, with no information on any relatives in the Register, or incomplete autoantibody analyses were excluded. Sex distribution or the median age of those included and excluded did not differ.

Study III

For the study III (CD autoimmunity), we compiled a series of 745 families with blood samples available from mother, father, at least one sibling, and an index child diagnosed with T1D before the age of 15. At the time of data collection in August 2007, the Register included a total of 2758 index children (median age at diagnosis 8.2 years, 56.1% male). The 745 index children who fulfilled the inclusion criteria were diagnosed at a median age of 8.8 (range 0.8-14.99) years and 56.6% were boys. In addition, we included 745 mothers (median age 38.3 years), 745 fathers (median age 40.5 years), and 1202 siblings (median age 9.7 years, 49.8% male) of the index children. Thus, the total number of study subjects was 3437.

Study IV

For the study IV (HLA and non-HLA genetics) we included children with genotyping data available for non-HLA loci. The genotyping was originally performed for a study involving trio families with samples from both parents and one affected child diagnosed with T1D under the age of 15 years [265]. Of these children, 1784 were included in the study IV. The median age at diagnosis was 7.9 years and 57% were male. During quality control, however, 39 individuals failed the missingness tests (genotyping call rate of at least 50%). The number of study subject for the final analysis was thus reduced to 1745.

Pooling of the study subjects

For the data presented in this thesis book, the study subjects included in different studies were pooled together. In addition to the 1488 children included in studies I and II, 40 children included only in study III and 726 children included only in study IV were added. One child was excluded since maturity onset diabetes of the young (MODY) was discovered in the family after the inclusion in the original study. Eight children were excluded because only one child per family was allowed in the study population. In general, the sibling included in study III was included, since this is the study with the most follow-up data collected from the family. Finally, this totaled 2245 index children with T1D diagnosed between January 2002 and November 2009. This time period coincides with the peak incidence of T1D in Finnish children [7]. If we assume 500-550

new cases of pediatric T1D in Finland annually, this thesis includes 51-56% of all children diagnosed during this time period. The majority were boys (n=1283, 57.1%) and the mean age at diagnosis of T1D was 7.9 years (median 8.1, range 0.28-15.0).

Methods

Determining family history of autoimmune diseases

Data from the structured questionnaires of the Register were the basis of family information on all studies. Diabetes of first-degree family members and grandparents was available from the structured part of the questionnaire, and information on other diseases and other relatives was manually coded from open answers. The extra information provided by the family varied from none to an extra sheet of paper. All this data was read and coded accordingly. For example, diabetes described to have developed after growth hormone and glucocorticoid therapy in the absence of islet autoantibodies was re-classified as other diabetes.

For studies I and II, only the first child registered in the Finnish Diabetes Register was included and all possible additional information provided by the the questionnaires of the successive family members diagnosed and registered after the diagnosis of the index case were omitted. Accordingly, these studies represent the situation at the time of T1D diagnosis.

In study III, data from the questionnaires was used to determine the frequency of known CD diagnoses at T1D diagnosis of the index children. To include as much follow-up data as possible, the prevalence of known CD and hypothyreosis diagnoses by April 2012 [median 7.7 (range 5.1-10.2) years after the T1D diagnosis of the index case] was based on data from the Social Insurance Institution of Finland. We obtained data for all 3437 study subjects on right for medicine expenses reimbursement due to hypothyreosis, right for dietary reimbursement due to CD, and right for disability allowance due to CD. Since this registration is a requirement for financial benefits, we assumed that most diagnoses were included. However, a child receiving disability allowance due to T1D will not qualify for additional benefits if diagnosed with CD. Accordingly, these new CD diagnoses might not have been recorded to the Social Insurance Institution. For this reason, the patient records were checked for children with T1D but without known CD

diagnosis. The subjects with high anti-tTG and/or EMA in our assays, were contacted/attempted to contact by local hospitals to offer current autoantibody testing and/or duodenal biopsy to diagnose CD.

In study IV, we included all available data on diseases of the index child and family members after the above mentioned methods of ascertainment. Additionally, for 42 children, more information on diseases of the family were acquired from the questionnaires filled in when an additional family member was diagnosed with diabetes and registered.

Thus when pooled together, around 800 of the 2245 children (36%) had follow-up data collected at a median of eight years after the diagnosis of the index case.

As data for other AIDs was collected with open questions in the questionnaire, the families used their own words to name the diseases of the family. The AIDs were grouped for data handling and analysis under autoimmune thyroid disease (hypothyreosis, hyperthyreosis of autoimmune origin), CD (CD and dermatitis herpetiformis), rheumatoid diseases and other AIDs. Rheumatoid diseases included e.g. rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus, Sjögren's syndrome, mixed connective tissue disease, scleroderma, collagenosis NAS, psoriatic arthritis, and polymyalgia rheumatica. Other AIDs included e.g. psoriasis, MS, pernicious anemia, adrenal dysfunction, inflammatory bowel diseases (Crohn's disease and ulcerative colitis), vitiligo, alopecia, iritis, Wegener's granulomatosis, and myasthenia gravis.

All available information was used for analyses (number of cases with missing information presented in Table 8). Missing information on diabetes status was structurally recorded in the questionnaire for first-degree relatives and grandparents. This data was taken into account when calculating for example prevalences of T1D in different relative groups in study I. When creating comparison groups, e.g. familial vs. sporadic T1D, the children with missing information on some relatives, and no T1D reported in any other relatives, were counted as sporadic cases. For the pooled analyses in this thesis book, however, this missing information was not accounted for. The rationale was to make the data easily comparable with that on studies II-IV. The differences in prevalences with or without missing information is at maximum two tenths of a percent. The information on other AIDs, however, was not collected structurally, and accordingly, we have no systematic information on those relatives with unkown disease status. Thus, all relatives not reported to specifically have a diagnosis of an AID in the questionnaire, were counted as not having any AIDs.

Grouping of cases

In study I, children with familial T1D were compared to sporadic cases. Familial cases were further subdivided to those with affected first-degree relatives (mother, father, sibling), second-degree relatives (grandparents, siblings of parents), or both.

In study II, the children who themselves had another AID in addition to T1D were compared to children with T1D only. The children with family history for other AIDs (those with family history for T1D excluded) were compared to a group of children with T1D only and without any first- or second-degree relatives with T1D or other AIDs. A classification of children to so called autoimmune families was also used. An autoimmune family was defined as more than three (≥4) diagnoses of AIDs (T1D or other) and/or more than two (≥3) different AIDs (T1D or other) in first- and/or second-degree relatives and/or the index child. This definition was elected to enable analysis of families were autoimmunity was truly clustered when at the same time ensuring adequate group size for statistical analysis. Thus, for the comparison of family history of other AIDs, the effect of familial T1D is completely excluded as these children are excluded from both comparison groups. In contrast, the comparison of autoimmune families studies differences between families with clustered autoimmunity for T1D or other AIDs.

In study III, children with CD and children with anti-tTG were compared to children without CD or anti-tTG. Similarly, first-degree relatives of children with T1D with CD and with anti-tTG were compared to relatives without CD or anti-tTG.

In study IV, genetic determinants were assessed between children with addional AIDs and children with T1D only, as well as children from autoimmune families and children with T1D only and without any family history of T1D or other AIDs.

When pooled together for this thesis, the compared groups were changed somewhat. Children with different forms of familial T1D or family history of other AIDs were both compared to a group of children with T1D only and without any family history for T1D or other AIDs (n=1089). This is in contrast to study I, in which familial cases were compared to cases without family history of T1D regardless of the family history for other AIDs. Children with additional autoimmune diseases were compared to children with T1D only and without anti-tTG (n=2152).

Table 8. *Number of cases with missing data according to variables used in the analyses. * x ≠ DR4-DQ8, † y ≠ DR3-DQ2*

Variable	Index children (n=2245)	Fathers	Mothers
Sex	0		
Age at diagnosis of the index child	1	755	723
Days from diagnosis at sample	5		
Number of children in the family	3		
Number of children with diabetes in the family	25		
Birth order of the index child	8		
First affected child of the family	24		
All data on diabetes of the core family missing	19		
Diabetes data of the father	58		
Diabetes data of the mother	31		
Diabetes data of the siblings	27		
Diabetes data of brothers	28		
Diabetes data of sisters	28		
Diabetes data of all grandparents	53		
Diabetes data on paternal relatives	35		
Diabetes data on maternal relatives	8		
Islet cell autoantibodies (ICA)	6		
Insulin autoantibodies (IAA)	7		
IA-2 autoantibodies (IA-2A)	7		
GAD autoantibodies (GADA)	7		
ZnT8 autoantibodies (ZnT8A)	333		
HLA risk group	14		
DR3-DQ2/DR4-DQ8	31		
DR3-DQ2/x*	28		
DR4-DQ8/y†	32		
x*/y†	22		
Protective genotype	24		
DR4-DQ8 positive	27		
DR3-DQ2 positive	28		
DR3-DQ2 homozygote	22		
DR4-DQ8 homozygote	23		
Blood glucose	34		
pH	61		
Betahydroxybutyrate	389		
Level of consciousness	96		
Duration of symptoms, days	1115		
Duration of symptoms, categorical	251		
Weight loss	10		
Height, cm	44		
Weight, kg	31		
BMI	46		

Autoantibody assays

IAA, GADA, IA-2A, and ZnT8A were analyzed using specific radiobinding assays [46, 266-268]. The cut-off limits were 2.80, 5.36, 0.77, and 0.61 RU, respectively. These were determined as the 99th percentiles in Finnish non-diabetic children and adolescents. According to the 2010 Diabetes Autoantibody Standardization Program (DASP), the sensitivities of these assays were 50, 88, 64 and 60% and specificities 96, 94, 99 and 100%. Islet cell antibodies (ICA) were analyzed with indirect immunofluorescense on human group 0 donor pancreas with 2.5 Juvenile Diabetes Foundation (JDF) units as the detection limit [17]. Only samples at or above the cut-off for antibody positivity were included when calculating the median antibody titers. Samples that were taken more than 30 days after the diagnosis of T1D were excluded from the antibody analyses.

In study III, all 3437 samples were analyzed for total- and IgA-specific anti-tTG. The radiobinding immune assay for total-anti-tTG is essentially like those for islet autoantibodies and measures mostly IgG isotype. The IgA-specific anti-tTG assay was similar to that described previously for isotype-specific β-cell autoantibodies [269]. The cut-off limit for total-anti-tTG positivity was 1.75 RU and for IgA-anti-tTG 1.56 RU, representing the 99th percentile in 216 healthy Finnish children and adolescents. According to the 2007 anti-tTG workshop, the disease sensitivity and specificity of the IgA-anti-tTG assays were 91% and 98%, respectively. To rule out IgA deficiency, the samples negative for IgA- but positive for total-anti-tTG were analyzed with a photometric, immunochemical method for total IgA levels in the Helsinki University Central Hospital Diagnostic Laboratory (HUSLAB). EMA were analyzed in Tartu with an immunofluorescence method described previously [270]. Cut-off for positivity was serum dilution 1:5.

Markers of metabolic decompensation at diagnosis

Blood pH, plasma glucose, and plasma beta-hydroxybutyrate levels of the index children were analyzed in local laboratories at diagnosis of T1D.

Genetic analyses

Analysis of HLA genotypes

Polymerase chain reaction (PCR) amplification of gene segments followed by hybridization with lanthanide-labeled sequence-specific oligonucleotide probes using time resolved fluorometry for detection was used for HLA typing of major T1D risk DR-DQ haplotypes [51]. At the time of analysis, a risk classification to five groups according to HLA class II genotyping results was used for the Register. Three groups represent those with an increased risk of T1D (risk over 1% by 15 years of age, groups 2-4), one group is neutral (riskgroup 1) and the last protects from T1D (riskgroup 0).

Analysis of non-HLA genotypes

The Sequenom (San Diego, California, USA) platform (Genome Center of Eastern Finland, University of Eastern Finland, Kuopio) was used for genotyping non-HLA SNPs in study IV. CCR5 delta-32 deletion (rs333) was genotyped using PCR with fluorescently labelled primers and automated sequencer.

Statistical methods

SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) versions 17.0 through 24 were used for the majority of the analyses. Differences in frequencies were evaluated with cross-tabulation and χ^2 statistics with Yates continuity correction or Fisher exact test when appropriate. For evaluation of differences in continuous variables Student's t-test or one way ANOVA were used for normally distributed variables, and Mann-Whitney U test/Wilcoxon's rank sum test for two groups or Kruskal-Wallis test with Dunn's post hoc test for over two groups of comparison for skewed distributions. Linear trend for variables with over two groups was tested with linear-by-linear test for categorical variables and Jonckheere-Terpsta test for continuous variables. For binary outcome variables logistic regression was used when adjusting for confounding factors and for continuous outcome variables with a skewed distribution quantile regression in R 12.2.1 package for statistical computing, package quantreg 4.54.

For study IV, association analysis with PLINK v1.07 software package (http://pngu.mgh.harvard.edu/purcell/plink/) [271] was used for testing allele associations with phenotypic traits. Power calculations for the discussion in Study IV were carried out with Quanto 1.2.4 (http://hydra.usc.edu/gxe, 2006) software. For multivariate analyses, standard regression models were built to assess the contribution of HLA and non-HLA genes. Generalized linear models with complementary log-log link functions and stepwise forward model selection to minimize Akaike's information criterion were applied.

A two tailed P-value of 0.05 or less determined statistical significance. In general, Bonferroni corrections were not applied due to their overly conservative nature. In study IV, the P values of association analyses were corrected for multiple comparisons by false discovery rate (FDR) step-up procedure described by Benjamini and Hochberg [272]. In general, the increased probability of falsely positive statistical tests from a large number of tests, was taken into account by cautious interpretation of the results.

Ethics

The ethics committee of the Hospital district of Helsinki and Uusimaa has approved the protocol of the Finnish Diabetes Register and Sample Repository as well as the ancillary study of related CD autoimmunity. All participants aged 10 to 17 years give their written assent for participation and the legal guardians of the children and all participants over the age of 18 years give their written informed consent at the time of registration. The study was conducted according to the principles of the declaration of Helsinki.

Results

For the 2245 index children, median number of days from the diagnosis to drawing of the blood sample was 5 (range -301-1385). Almost all children (98%) had at least one positive T1D related autoantibody. Median number of positive autoantibody responses was four out of five. According to the data provided to the Register, 439 (19.6%) children did not have any siblings.

Frequency of autoimmune diseases

Index children

At diagnosis of T1D, 37 (1.6%) children had already been diagnosed with another AID. At the end of the study with a median of 8 years of follow-up, 71 (3.2%) had some additional AID (Table 9), and 34 (1.5%) had CD. Of the children with T1D only (n=2174), 701 children were included in study III and were thus analyzed for anti-tTG at diagnosis of T1D. Of these, 22 had anti-tTG. These children were excluded from the comparison group making the number of children in the group of no additional autoimmunity 2152. There was a trend for more AIDs in those children older at T1D diagnosis; prevalence of additional AIDs was 2.3% among 0-4-year-olds, 2.8% among 5-9 year-olds, and 4.2% among 10-15 year-olds (P for trend=0.04).

Of the 2245 children, 744 children were included in Study III (one child originally included in this study was excluded as MODY was discovered) and were analysed for anti-tTG at diagnosis of T1D and extensively investigated for the presence of CD. Of these 36/744 (4.8%) had anti-tTG. Of the anti-tTG positive, 36.1% (13/36) were also positive for EMA.

CD at diagnosis of T1D was reported by 0.7% (5/744) of the index cases, and, after anti-tTG screening and search through registers of the Social Insurance Institution of Finland and patient records, 24 (3.2%) had CD. Interestingly, only 36% (13/36) of those with anti-tTG at diagnosis had developed CD by the final search [or 13 of the 24 CD patients (54%) had anti-tTG at diagnosis of T1D]. This means that 64% (23/36) of the index children with anti-tTG at diagnosis of T1D did not develop CD even after median

follow-up of eight years, despite seven of these (7/23, 30%) having either high anti-tTG (≥10 times the upper limit of normal) or positive EMA.

Table 9. *Number of index children with other autoimmune diseases in addition to T1D at diabetes diagnosis and at the end of the study. *At diagnosis one child had both AIT and rheumatoid disease, and at the end of the study two more children had multiple diagnoses: one with AIT and CD, and another with CD and vitiligo.*

Disease	At diagnosis of T1D	At the end of study
Celiac disease	15	32
Autoimmune thyroid disease	12	27
Rheumatoid disease	5	5
Colitis ulcerosa	1	1
Alopecia	2	2
Vitiligo	1	1
Two different diseases*	1	3
Total	37/2245 (1.6%)	71/2245 (3.2%)

Familial type 1 diabetes

At diagnosis of T1D, 264 (11.8%) of the 2245 children had a first-degree relative with T1D. At the end of the study this figure had increased to 305 (13.6%) (Figures 1 and 2). Of these children, 27 (1.2%) had two affected first-degree relatives, two (0.1%) had three, and one had four (mother, father, two sisters). The number of affected fathers was 134 (6.0%). The number of mothers with T1D was 73 (3.3%) at diagnosis, and one additional diagnosis was discovered by the end of the study: 74/2245 (3.3%). The number of affected brothers increased from 42 (42/2245, 1.9%) to 65 (2.9%), and affected sisters from 33 (1.5%) to 60 (2.7%) from diagnosis to the end of study. This increased the number of affected siblings from 72 (3.2%) to 122 (5.4%) (the gender of one affected sibling was not reported). In other words, of those 1803 children known to have siblings, 3.9% at diagnosis and 6.8% at the end of study had affected siblings. The maximum

number of affected siblings was three. Prevalence of T1D among the total of 3101 siblings in the cohort, was 4.3% (132/3101).

The frequency of affected second-degree relatives was the same at diagnosis and at the end of study; 281 (12.5%). The number of children with affected maternal second-degree relatives was 156 (6.9%) and affected paternal relatives 138 (6.1%). The number of affected second-degree relatives ranged from zero to five.

In total, 489 (21.8%) children had a relative with T1D at diagnosis of T1D, and at the end of study the number was 522 (23.3%). Fifty-six children (2.5%) had both a first- and second-degree relative affected at diagnosis, and at the end of the study, 64 (2.9%). The maximum number of affected relatives in the extended family at the end of the study was six. The total number of second-degree relatives is unknown.

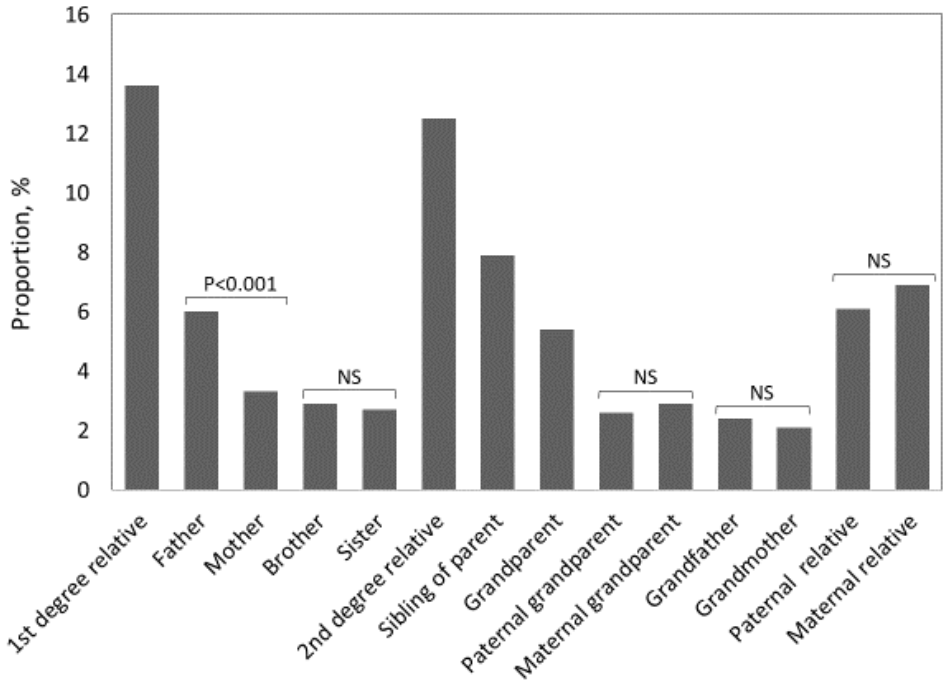

Figure 1. *Proportions (%) of families who reported T1D in different relative groups. Results for test for binomial proportions are shown.*

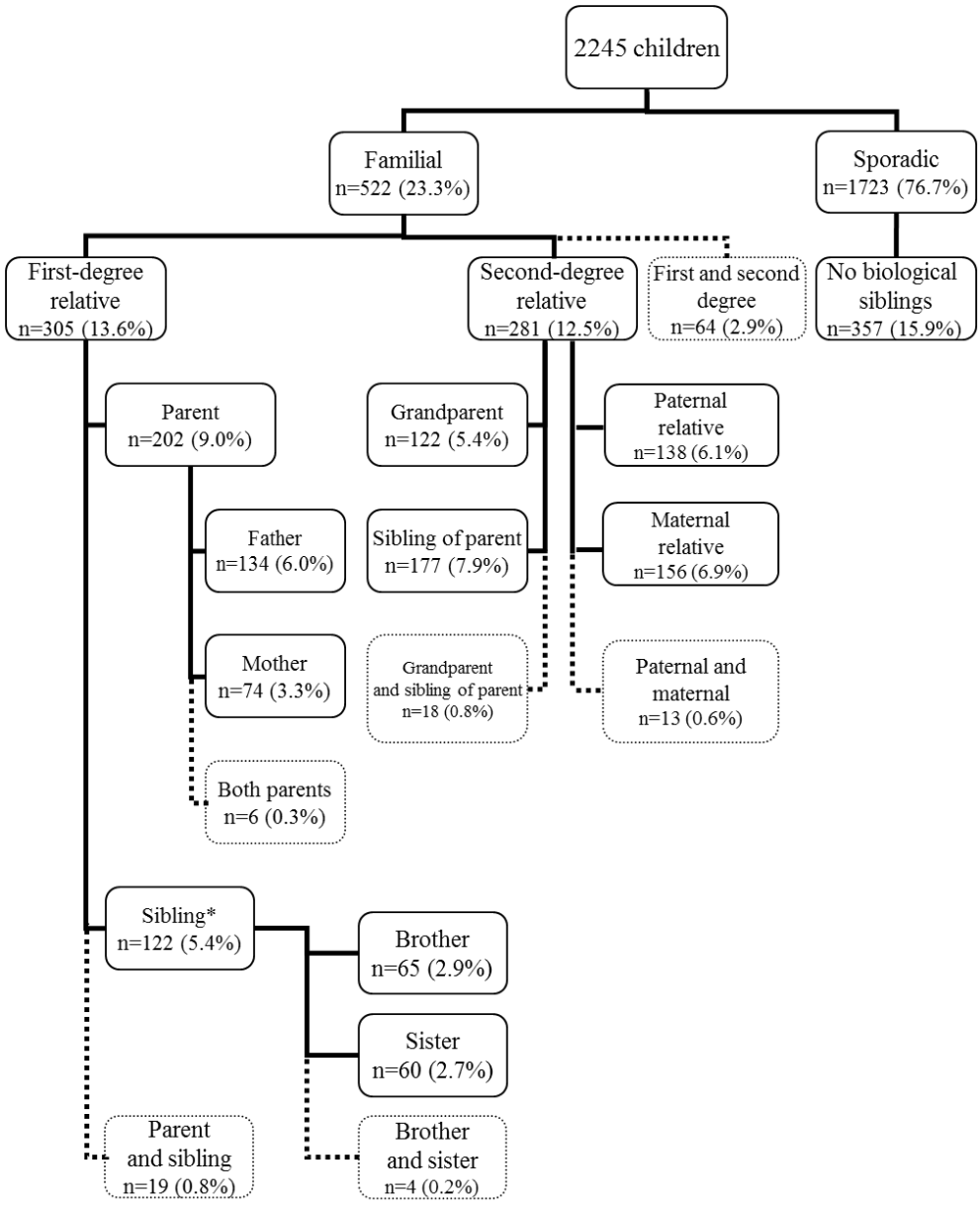

Figure 2. *Flowchart on familial T1D showing the number and proportion of the total 2245 children for children with T1D patients in different relative groups. *Gender of one affected sibling was not reported.*

Other autoimmune diseases

At diagnosis of T1D, 307 (13.7%) index children had a first-degree relative affected with an AID other than T1D. At the end of study, this figure was 331 (14.7%; Figures 3 and 4). More mothers than fathers had AIDs (Figure 4); at diagnosis 195 (8.7%) mothers had an AID compared to 72 (3.2%) fathers, and at the end of the study 223 (9.9%) mothers had an AID in contrast to 86 (3.8%) fathers. At diagnosis, 23 (1.0%) children reported having a sibling with an AID other than T1D, and at the end of study 40 (1.8%). Of those 1803 children reported to have siblings, these figures translate to 1.2% and 2.2%, respectively. The prevalence of other AIDs was 1.4% (44/3101) of all 3101 siblings.

For second-degree relatives the figures did not increase during the study period; 590 (26.3%) children reported a second-degree relative with an AID other than T1D. In total, 499 (22.2%) of families reported affected grandparents; 405 (18.0%) families had affected grandmothers, and 134 (6.0%) families affected grandfathers. Affected paternal second-degree relatives were reported by 282 (12.6%) families, and affected maternal second-degree relatives by 393 (17.5%) families. The total number of second-degree relatives is not known, which prevents us from reporting actual prevalences of diseases except for grandparents; the prevalence of AID other than T1D was 9.0% (405/4490) for grandmothers, 3.0% (134/4490) for grandfathers.

In total, at diagnosis 760 (33.9%) and at the end of the study 804 (35.8%) index children had AID other than T1D in the extended family (first or second-degree relatives).

At the end of the study, AIT in the extended family was reported by 432 (19.2%) families [at diagnosis 424 (18.9%)], CD by 177 (7.9%) families [at diagnosis 128 (5.7%)], rheumatoid disease by 291 (13.0%) of families, and other AIDs by 107 (4.8%) of the families. These diseases were more common among second-degree than first-degree relatives, with the exception of CD since first-degree relatives were specifically screened for this disease in study III (Figure 5). The maximum number of first and/or second-degree relatives affected by AIT was 8, by CD was 4, by rheumatoid diseases was 4, and by other AIDs was 3. In total, 270 (12.0%) children fulfilled the criteria for an autoimmune family.

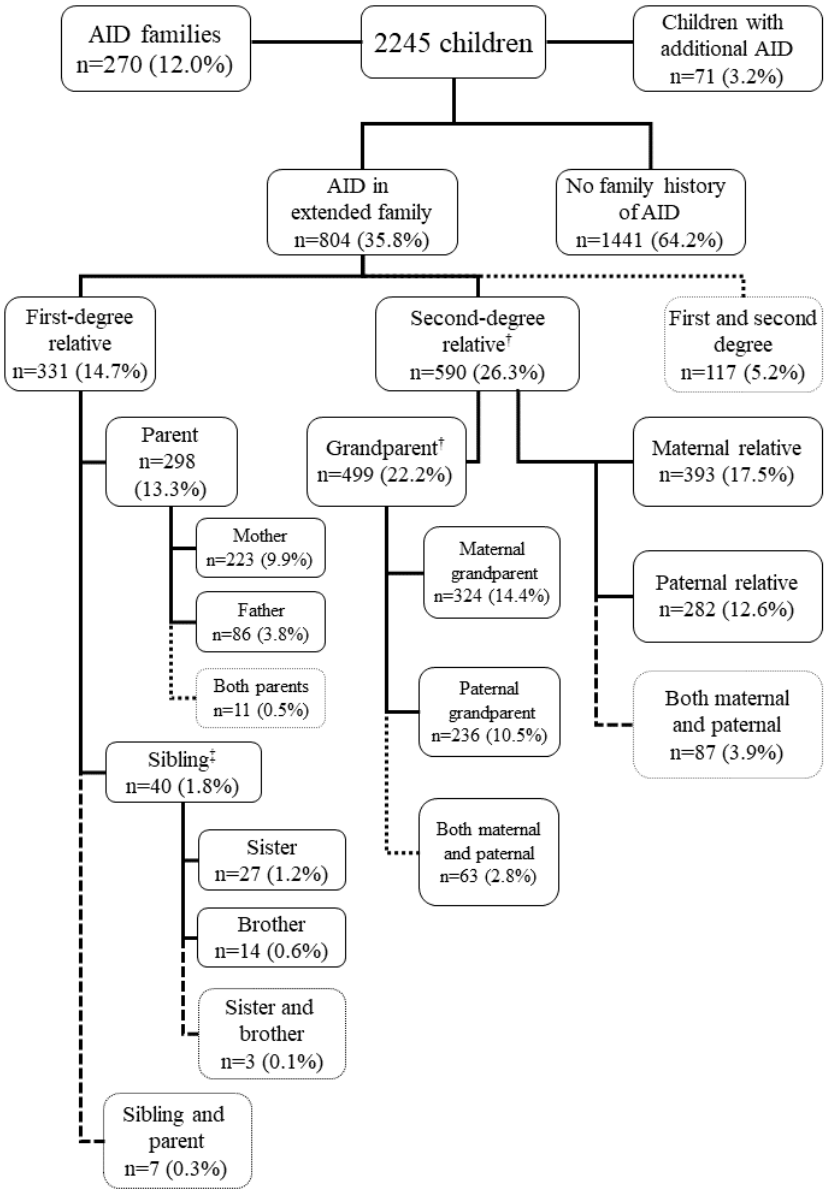

Figure 3. *Flowchart on familial clustering of autoimmune diseases other than T1D showing the number children and the proportion of the total 2245 children for children with affected relatives from different relative groups. †For two affected grandparents the side of family (paternal/maternal) was not reported. ‡Gender of two affected siblings was not reported.*

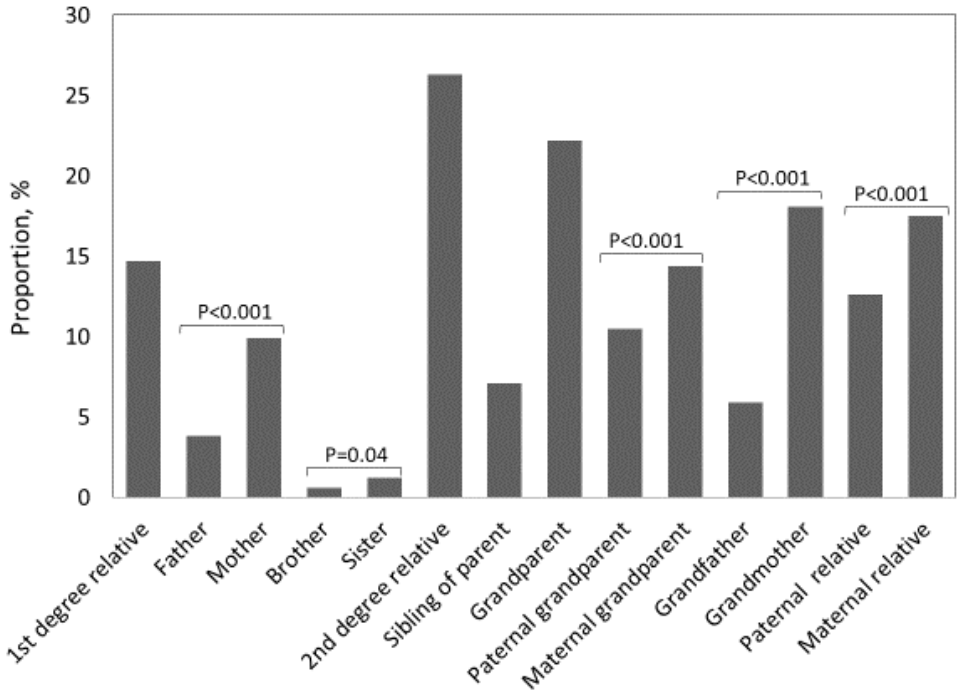

Figure 4. *Proportions (%) of families who reported AIDs other than T1D in different relative groups. Results for test for binomial proportions are shown.*

The children with additional AIDs (n=71) or the children with extended family members with AIDs other than T1D (n=804), did not have relatives with T1D more often than children with T1D only (22.5 vs. 23.3%, NS) or children with no family history of autoimmune diseases (24.4 vs. 22.6%, NS).

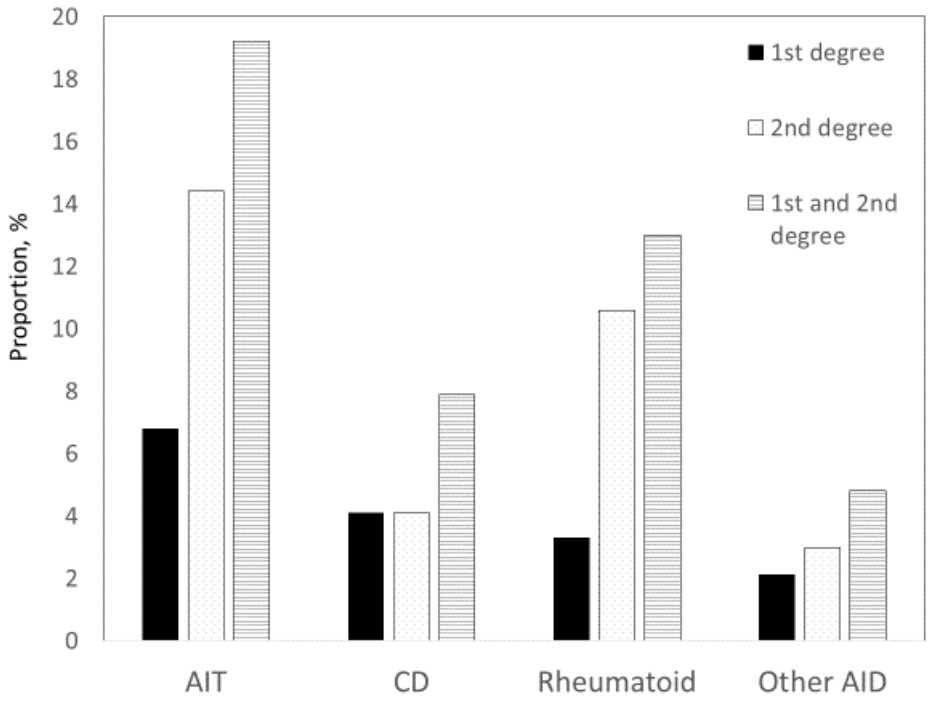

Figure 5. *Proportions of index children with relatives affected by AIDs in first- or second-degree relatives or both. AIT=autoimmune thyroiditis, CD = celiac disease.*

In total, 2689 first-degree relatives of 744 children were included in Study III (three relatives of the child later discovered to have MODY were excluded). Among these relatives, the prevalence of anti-tTG positivity in the sample at T1D diagnosis of the index child was 2.9% (79/2689); 4.8% (36/744) among the mothers, 2.4% (18/744) among the fathers and 2.1% (25/1201) among the siblings (Figure 2 in study III). Of the 79 anti-tTG positives, 50 (63.3%) had also EMA. The respective proportions were 66.7% (24/37) for the mothers, 72.2% (13/18) for the fathers, and 52.0% (13/25) for the siblings. Only 0.6% (15/2689) of the relatives reported CD in the Register [1.1% (8/745) of the mothers, 0.5% (4/745) of the fathers, and 0.2% (3/1202) of the siblings]. By the end of case ascertainment, 2.5% (67/2689) had CD; 34 (4.4%) mothers, 16 (2.1%) fathers, and 17 (1.4%) siblings (Figure 2 in study III). Of the 79 anti-tTG positive relatives, 48 (60.1%) had CD by the end of data collection (or 48 of the 67 CD patients (72%) had anti-tTG in the sample at diagnosis of the index child). This means that 39% (31/79) of

anti-tTG positive relatives (33% of mothers, 28% of fathers, 56% of siblings) did not develop CD after eight years of follow-up, although 11 (35%, 11/31) of them had either high anti-tTG levels (≥10 times the upper limit of normal) or positive EMA.

Characterizing familial type 1 diabetes

Demographics

The children with familial or sporadic T1D did not differ in terms of age at diagnosis or gender distribution (Study I, Table 10). The familial cases came from bigger families; the number of children in the family was higher, they were less often the only child, and the birth order of the child (first, second, third etc. child of the family) was higher. The size of the family relates to age at diagnosis, as the children who were the only child of the family were younger at diagnosis (median 5.8 vs. 8.6 years yrs, $P<0.001$).

The frequency of familial T1D did not differ according to age groups; affected relatives in extended family were reported in 14.8% of 0-5-year-olds, 13.9% of 5-10-year-olds, and 13.6% of 10-15-year-olds, $P=0.81$. Girls with maternal second-degree relatives with T1D were younger than girls with paternal second-degree relatives with T1D (6.4 vs. 8.1 years, $P=0.04$, Table 11).

Table 10. *Demographics, markers of metabolic decompensation at diagnosis, autoantibodies, and HLA class II genetics of children with extended family members (first- and/or second-degree relatives) affected with T1D (familial T1D) compared to children with no family history of T1D or other autoimmune diseases. *x≠DR4-DQ8 †y≠DR3-DQ2*

	I. Familial T1D (n=522)	II. No additional AID in family (n=1089)	P value
Demographics			
Sex, male, %	58.0	57.6	0.90
Age at diagnosis, yrs, median (range)	7.61 (0.28-14.95)	8.32 (0.69-15.0)	0.09
Number of children in the family, median (mean)	2 (2.6)	2 (2.3)	**<0.001**
Only child, %	16.3	23.3	**0.002**
Birth order, median (mean)	2 (1.9)	1 (1.7)	**0.004**
Number of days from diagnosis at sample, median	5	5	0.40
Metabolic decompensation at diagnosis			
Blood glucose, mmol/l, median (range)	22.9 (3.6-97.6)	25.0 (4.9-92.7)	**<0.001**
Ketoacidosis, %	9.8	20.7	**<0.001**
pH, median (range)	7.39 (6.80-7.54)	7.37 (6.84-7.52)	**<0.001**
ß-hydroxybutyrate, mmol/l, median (range)	0.9 (0-20.1)	2.1 (0-16.2)	**<0.001**
Impaired consciousness, %	2.2	6.0	**0.002**
Duration of symptoms, days, median (range)	7 (0-332)	9 (0-314)	0.24
Weight loss, kg, median, (range)	0.60 (0-15.0)	1.5 (0-17.0)	**<0.001**
BMI (range)	15.8 (11.1-32.7)	15.5 (11.0-32.7)	**0.005**
Autoantibodies			
ICA, %	90.2	93.5	**0.03**
ICA, JDFU, median (range)	40 (3-1,517)	40 (3-39,935)	0.42
IAA, %	44.0	42.1	0.52
IAA, RU, median (range)	10.7 (3.0-282.1)	10.3 (2.9-386.1)	0.76
IA-2A, %	73.8	75.0	0.65
IA-2A, RU, median (range)	103.3 (1.2-256.4)	101.9 (0.86-501.0)	0.09
GADA, %	65.6	67.2	0.57
GADA, RU, median (range)	36.3 (5.4-1,699.2)	36.2 (5.6-324.7)	0.38
ZnT8A, %	62.4	62.9	0.92
ZnT8A, RU, median (range)	5.9 (0.6-200.3)	6.4 (0.6-459.4)	0.38
Positive antibody responses, median (mean)	4 (3.4)	4 (3.4)	0.53

HLA class II genetics	I. Familial T1D (n=522)	II. No additional AID in family (n=1089)	P value
DR3-DQ2/DR4-DQ8, %	22.8	20.7	0.38
DR3-DQ2/x*, %	13.1	15.6	0.22
DR4-DQ8/y†, %	51.7	46.8	0.07
x*/y†, %	12.2	16.6	**0.03**
Protective genotype, %	2.5	3.4	0.40
DR3-DQ2 positive, %	36.0	36.4	0.90
DR4-DQ8 positive, %	74.7	67.7	**0.006**
DR3-DQ2 homozygote, %	3.7	2.4	0.21
DR4-DQ8 homozygote, %	10.8	5.8	**<0.001**

Table 11. *Age and HLA class II genetics in children with second-degree relatives with T1D from the maternal or paternal side of the family. *x≠DR4-DQ8 †y≠DR3-DQ2*

		Second-degree familial cases		
		I. Paternal (n=138)	II. Maternal (n=143)	P value
Age at diagnosis, yrs, median (range)	male	8.1 (1.5-15.0)	6.9 (0.8-14.7)	0.42
	female	8.1 (1.3-14.4)	6.4 (1.6-14.4)	**0.04**
HLA class II genetics				
DR3-DQ2/DR4-DQ8, %	male	9.1	30.0	**0.003**
	female	25.7	14.3	0.23
DR3-DQ2/x*, %	male	22.7	15.0	0.29
	female	11.4	2.4	0.15
DR4-DQ8/y†, %	male	53.0	47.0	0.55
	female	55.1	61.9	0.61
x*/y†, %	male	15.2	8.0	0.23
	female	7.2	21.4	0.06
Protective genotype, %	male	3.0	0	0.16
	female	0	4.8	0.14
DR3-DQ2 positive, %	male	31.8	45.0	0.12
	female	37.1	16.7	**0.04**
DR4-DQ8 positive, %	male	62.1	77.0	0.06
	female	81.2	76.2	0.70
DR3-DQ2 homozygote, %	male	3.0	3.0	1.00
	female	4.3	2.4	1.00
DR4-DQ8 homozygote, %	male	7.6	9.0	0.97
	female	18.6	11.9	0.51

There were gender differences among the cases in terms of who in the family had type 1 diabetes (Study I, Table 12). In general, boys and girls had equally often relatives affected with T1D, and for first-degree relatives there were no differences. For second-degree relatives, however, boys had more often affected relatives from the maternal side and girls from the paternal side.

Table 12. *Comparison of frequency of reporting T1D in different relative groups between boys and girls. The data are percentages (%) of the total number of male/female cases with information provided for the relative in question.*

Relative with T1D at the end of study	Boys, % n=1283	Girls, % n=962	P-value
Any relative	23.6	22.8	0.67
First-degree relative	13.6	13.6	1.00
Father	6.1	6.2	0.94
Mother	3.4	3.3	0.97
Brother	2.3	3.2	0.66
Sister	2.7	2.7	1.00
Second-degree relative	13.0	11.9	0.45
Paternal grandparent	1.8	4.0	**0.004**
Father's father	1.0	1.9	0.13
Father's mother	0.8	2.1	**0.02**
Maternal grandparent	3.7	2.3	0.09
Mother's father	2.4	1.3	0.10
Mother's mother	1.4	1.0	0.51
Sibling of father	3.7	4.1	0.70
Sibling of mother	5.0	3.3	0.07
Paternal second-degree	5.2	7.6	**0.03**
Maternal second-degree	8.1	5.4	**0.02**

Autoantibodies

Children with familial T1D were less often positive for ICA compared to children with sporadic T1D (Tables 10 and 13). This finding did not reach statistical significance in Study I. There were no differences in ICA titers, however. Children with two or more

relatives with T1D tended to have lower number of positive autoantibody responses (Table 13).

There were no differences in autoantibody profile in children from multigenerational families (parents with T1D) compared to children from multiplex families (siblings with T1D), or in children with T1D in maternal compared to paternal second-degree relatives.

HLA class II genetics

As observed in Study I, children with familial T1D had more often the HLA DR4-DQ8 haplotype compared to children without family history for AIDs (Table 10). In contrast to Study I, which had a smaller sample size and analyzed data only from the time of T1D diagnosis of the index case, the children with familial T1D at the end of study had also less often a genotype without either of the risk haplotypes (nonDR3-DQ2/nonDR4-DQ8) and were more often homozygous for DR4-DQ8. Especially the children with affected relatives in both first- and second-degree relatives (Table 13) and the children with two or more affected relatives (Table 14) had a conspicuously high frequency of DR4-DQ8 homozygozity.

Children form multigenerational of multiplex families did not differ in terms of HLA class II genetics. Children with affected second-degree relatives from maternal or paternal side of the family were compared separately for each sex (Table 11); girls with affected paternal second-degree relatives had more often the risk haplotype DR3-DQ2 and boys with maternal second-degree relatives the risk genotype DR3-DQ2/DR4-DQ8. A similar finding was reported in Study I, although statistical significance was reached for different haplotypes/genotypes. This finding concurs with the finding of more boys with affected maternal and girls with affected paternal second-degree relatives.

Table 13. *Comparison of children with first-degree, second-degree, or both relatives affected with T1D compared to children with no family history of AIDs. P-values for comparison of the four groups together are shown. For significant P-values in these comparisons, subgroup analyses with Dunn's post-hoc test were carried out and significant P-values are shown. P-value for trend (sporadic, first degree, second-degree, or both) is also shown. *x≠DR4-DQ8 †y≠DR3-DQ2*

	Familial T1D cases					
	I. First-degree (n=241)	II. Second-degree (n=217)	III. First and second-degree (n=64)	IV. No additional AID in family (n=1089)	*P* value	Test for trend: P value
Demographics						
Sex, male, %	56.4	59.4	59.4	57.6	0.92	
Age at diagnosis, yrs, median (range)	7.80 (0.3-14.95)	7.46 (0.8-14.95)	7.44 (1.3-14.6)	8.32 (0.69-15.0)	0.40	
Number of children in the family, median (mean)	2 (2.57)	2 (2.53)	2.5 (2.98)	2 (2.3)	**<0.001**	**<0.001**
					I vs. IV: 0.05	
Only child, %	16.2	16.6	15.6	23.3	**0.02**	**0.004**
					I vs. IV: 0.02	
					II vs. IV: 0.04	
Birth order, median (mean)	2 (1.93)	2 (1.90)	2 (2.14)	1 (1.7)	**0.04**	**0.006**
Number of days from diagnosis at sample, median	5	6	4	5	0.11	
Autoantibodies						
ICA, %	89.2	93.0	84.2	93.5	**0.01**	**0.03**
					I vs. IV: 0.04	
					III vs. IV: 0.01	
ICA, JDFU, median (range)	40 (4-759)	40 (3-1,517)	24 (3-759)	40 (3-39,935)	0.74	
IAA, %	46.8	42.3	38.6	42.1	0.54	
IAA, RU, median (range)	11.9 (3.1-145.2)	9.9 (3.0-282.1)	10.9 (3.2-190.7)	10.3 (2.9-386.1)	0.84	
IA-2A, %	75.7	74.6	63.2	75.0	0.25	
IA-2A, RU, median (range)	103.1 (1.2-202.6)	101.3 (1.3-256.4)	115.3 (4.2-177.7)	101.9 (0.86-501.0)	0.19	
GADA, %	64.4	68.2	61.4	67.2	0.67	
GADA, RU, median (range)	37.4 (5.4-394.9)	32.6 (5.4-1,699.2)	38.7 (8.9-267.1)	36.2 (5.6-324.7)	0.70	
ZnT8A, %	62.7	63.0	59.2	62.9	0.97	
ZnT8A, RU, median (range)	6.0 (0.6-200.3)	5.9 (0.8-165.0)	5.0 (0.7-85.2)	6.4 (0.6-459.4)	0.72	
Positive antibody responses, median (mean)	4 (3.4)	4 (3.4)	3 (3.1)	4 (3.4)	0.49	

Table 13. *Continued.*

	I. First-degree (n=241)	II. Second-degree (n=217)	III. First and second-degree (n=64)	IV. No additional AID in family (n=1089)	*P* value	Test for trend: P value
Metabolic decompensation at diagnosis						
Blood glucose, mmol/l, median (range)	22.9 (3.6-63.7)	23.1 (5.1-97.6)	22.2 (5.6-63.1)	25.0 (4.9-92.7)	**<0.001**	**<0.001**
					I vs. IV: 0.01	
					II vs. IV: 0.02	
Ketoacidosis, %	9.9	11.2	4.9	20.7	**<0.001**	**<0.001**
					I vs. IV: <0.001	
					II vs. IV: 0.002	
					III vs. IV: 0.004	
pH, median (range)	7.39 (6.80-7.54)	7.39 (6.90-7.53)	7.40 (7.05-7.48)	7.37 (6.84-7.52)	**<0.001**	**<0.001**
					I vs. IV: 0.004	
					II vs. IV: 0.03	
					III vs. IV: 0.01	
ß-hydroxybutyrate, mmol/l, median (range)	0.7 (0-20.1)	1.11 (0-11.0)	0.40 (0-9.8)	2.1 (0-16.2)	**<0.001**	**<0.001**
					I vs. IV: <0.001	
					II vs. IV: 0.05	
					III vs. IV: 0.01	
Impaired consciousness, %	1.7	2.9	1.7	6.0	**0.01**	**0.005**
					I vs. IV: 0.01	
Duration of symptoms, days, median (range)	7 (0-332)	8 (0-143)	7.5 (0-132)	9 (0-314)	0.15	
Weight loss, kg, median, (range)	0.5 (0-15.0)	1.0 (0-13.5)	0.25 (0-10.0)	1.5 (0-17.0)	**<0.001**	**<0.001**
					I vs. IV: <0.001	
					III vs. IV: 0.02	
BMI (range)	15.9 (11.8-32.7)	15.5 (11.1-26.4)	16.2 (12.5-26.2)	15.5 (11.0-32.7)	**0.001**	**0.008**

Table 13. *Continued.*

	I. First-degree (n=241)	II. Second-degree (n=217)	III. First and second-degree (n=64)	IV. No additional AID in family (n=1089)	*P* value	Test for trend: P value
HLA class II genetics						
DR3-DQ2/DR4-DQ8, %	24.3	20.9	23.8	20.7	0.64	
DR3-DQ2/x*, %	12.1	13.5	15.9	15.6	0.51	
DR4-DQ8/y†, %	50.6	54.0	48.4	46.8	0.24	
x*/y†, %	12.9	11.6	11.3	16.6	0.15	
Protective genotype, %	3.8	0.9	3.2	3.4	0.25	
DR3-DQ2 positive, %	36.4	34.4	39.7	36.4	0.89	
DR4-DQ8 positive, %	75.0	74.9	72.6	67.7	**0.04**	**0.02**
					I vs. IV: <0.04	
					II. vs. IV: 0.05	
DR3-DQ2 homozygote, %	4.2	4.2	0	2.4	0.14	
DR4-DQ8 homozygote, %	10.0	8.8	20.6	5.8	**<0.001**	**<0.001**
					I vs. III: 0.04	
					I vs. IV: 0.02	
					II vs. III: 0.02	
					III vs. IV: <0.001	

Table 14. *Comparison of demographics, autoantibodies and HLA class II genetics in children with two or more T1D patients in the extended family and children without family history of autoimmune diseases. *x≠DR4-DQ8 †y≠DR3-DQ2*

	I. Two or more affected relatives (n=113)	II. No additional AID in family (n=1089)	P value
Demographics			
Sex, male, %	57.5	57.6	1.00
Age at diagnosis, yrs, median (range)	8.25 (0.7-14.6)	8.32 (0.69-15.0)	0.36
Autoantibodies			
ICA, %	87.0	93.5	**0.03**
ICA, JDFU, median (range)	25 (3-759)	40 (3-39,935)	0.39
IAA, %	36.0	42.1	0.29
IAA, RU, median (range)	11.0 (3.2-190.7)	10.3 (2.9-386.1)	0.97
IA-2A, %	69.0	75.0	0.23
IA-2A, RU, median (range)	107.5 (1.9-203.3)	101.9 (0.86-501.0)	0.15
GADA, %	62.0	67.2	0.34
GADA, RU, median (range)	36.6 (5.4-267.1)	36.2 (5.6-324.7)	0.74
ZnT8A, %	59.5	62.9	0.63
ZnT8A, RU, median (range)	6.4 (0.7-165.0)	6.4 (0.6-459.4)	0.68
Positive antibody responses, median (mean)	3 (3.12)	4 (3.4)	**0.05**
HLA class II genetics			
DR3-DQ2/DR4-DQ8, %	22.5	20.7	0.75
DR3-DQ2/x*, %	14.4	15.6	0.85
DR4-DQ8/y†, %	54.5	46.8	0.15
x*/y†, %	8.2	16.6	**0.03**
Protective genotype, %	1.8	3.4	0.52
DR3-DQ2 positive, %	36.9	36.4	1.00
DR4-DQ8 positive, %	77.3	67.7	0.051
DR3-DQ2 homozygote, %	1.8	2.4	1.00
DR4-DQ8 homozygote, %	17.1	5.8	**<0.001**

Metabolic decompensation at diagnosis

Children with familial T1D had a milder metabolic decompensation at diagnosis than children with no family history of autoimmune diseases (Study I, Tables 10 and 13); they had lower blood glucose level, higher pH, lower levels of β-hydroxybutyrate, lower frequencies of ketoacidosis and impaired conciousness, lower weight loss and higher

BMI. Importantly, these differences were evident also in the group of children with only second-degree relatives with T1D (Study I, Table 13), indicating that it is not necessary for the other patient with T1D in the family to be from the immediate family. Those children with T1D in both first- and second-degree relatives (Table 13) seemed to have the best metabolic situation at diagnosis. The duration of symtoms was shorter in familial cases but the difference was not statistically significant.

There were no marked differences between children with affected paternal or maternal second-degree relatives, or children with multigenerational or multiplex T1D.

Characterizing T1D associated with other autoimmune diseases

Index children with additional autoimmune diseases

Demographics

Children with additional autoimmune diseases (n=71) were compared to children with T1D only and no anti-tTG (n=2152). As celiac disease was specifically screened for in a subset of cases in the context of Study III, and other diseases were not, comparison with children with CD excluded was also carried out (37 children compared to 2152, Table 15). Gender distribution did not differ significantly for either of these groups although there was a female majority in the groups of additional autoimmunity. The prevalence of additional autoimmune disease at the end of study was 4.0% among girls and 2.6% among boys (P=0.09). Children with AIDs other than CD were older at T1D diagnosis (Table 15). There was some tendency for more children with additional AIDs in the oldest age group; the prevalence was 2.3% among those diagnosed at 0-4 years of age, 2.8% among 5-9-year-olds, and 4.2% among 10-15-year-olds (P=0.09). Children with T1D only were more often the only child of the family (Table 15).

Autoantibodies

ICA positivity was more common among children with T1D only than in children with any additional AID or children with other AID than CD (Table 15). This difference was also seen in Study II. In addition, at the end of study, children with T1D only had more often IA-2A and ZnT8A, and had a higher number of positive autoantibody responses

than children with any additional AIDs. These differences disappeared, however, when children with CD were excluded (Table 15).

HLA class II genetics

In Study II, no differences in HLA class II genetics were evident between children with multiple AIDs or T1D only. At the end of study, with addition of many patients with CD and AIT, more genotypes and haplotypes differed between the groups; for children with any additional AID, clear overrepresentation of the DR3-DQ2 haplotype and underrepresentation of the DR4-DQ8 haplotype was evident (Table 15, Table 2 in study IV). When children with CD were excluded, children without either of these haplotypes and children with genotypes protective for T1D were overrepresented (Table 15).

Metabolic decompensation at diagnosis

In study II, with only children with known additional AID already at diagnosis of T1D included, evidence for milder metabolic decompensation (lower plasma glucose and β-hydroxybutyrate levels, higher pH, longer duration of symptoms) in children with multiple AIDs was observed. At the end of study, with inclusion of children who developed additional AIDs during follow-up after T1D diagnosis, such differences were no longer evident. Only longer duration of symptoms in children with additional AIDs was observed (Table 15).

Table 15. *Demographics, markers of metabolic decompensation at T1D diagnosis, islet autoantibodies, and HLA class II genetics in children with any additional AIDs or additional AIDs other than CD compared to children with T1D only and no tissue transglutaminase antibodies at diagnosis of T1D.*

	I. Children with additional AID (n=71)	II. Children with AID other than CD (n=37)	III. Children with celiac disease (n=34)	IV. Chilren with no additional autoimmunity (n=2152)	I vs. IV: P value	II vs. IV: P value	III vs. IV: P value
Demographics							
Sex, male, %	46.5	43.2	50.0	57.6	0.08	0.11	0.47
Age at diagnosis, yrs, median (range)	9.6 (1.2-14.99)	10.1 (1.8-14.99)	9.1 (1.2-12.2)	8.0 (0.3-15.0)	0.10	**0.05**	0.72
Number of children in the family, median (mean)	2 (2.54)	2 (2.49)	2 (2.59)	2 (2.37)	0.07	0.17	0.25
Only child, %	7.0	10.8	2.9	20.3	**0.009**	0.22	**0.02**
Birth order, median (mean)	2 (1.92)	2 (1.95)	2 (1.88)	2 (1.80)	0.06	0.08	0.35
Number of days from diagnosis at sample, median	6	6	7	5	0.16	0.86	0.06
Metabolic decompensation at diagnosis							
Blood glucose, mmol/l, median (range)	23.9 (6.0-44.7)	22.2 (13.3-42.0)	24.1 (6.0-44.7)	24.3 (3.2-97.6)	0.51	0.67	0.61
Ketoacidosis, %	16.9	13.5	20.6	17.4	1.00	0.69	0.80
pH, median (range)	7.39 (7.04-7.51)	7.39 (7.15-7.51)	7.39 (7.04-7.50)	7.38 (6.80-7.54)	0.14	0.19	0.42
Betahydroxybutyrate, mmol/l, median (range)	1.5 (0-11.6)	1.6 (0-8.5)	1.4 (0.05-11.6)	1.7 (0-20.1)	0.56	0.96	0.36
Impaired consciousness, %	1.6	0	3.4	4.8	0.36	0.40	1.00
Duration of symptoms, days, median (range)	19.5 (0-183)	23.5 (0-183)	15 (1-114)	9 (0-377)	**0.01**	**0.009**	0.26
Weight loss, kg, median (range)	1.2 (0-12.1)	2.0 (0-10.0)	1.0 (0-12.1)	1.0 (0-20.0)	0.94	0.20	0.22
BMI, median (range)	15.2 (12.0-27.3)	15.7 (12.0-27.3)	14.7 (12.8-21.8)	15.6 (10.2-33.6)	0.07	0.80	**0.02**

Table 15. *Continued.*

	I. Children with additional AID	II. Children with AID other than CD	III. Children with celiac disease	IV. Chilren with no additional autoimmunity	I vs. IV: p value	II vs. IV: p value	III vs. IV: p value
Autoantibodies							
ICA, %	81.2	75.7	87.5	93.2	**0.001**	**0.001**	0.27
ICA, JDFU, median (range)	40 (4-640)	40 (4-335)	23 (4-640)	40 (2.5-39,935)	0.69	0.46	0.19
IAA, %	37.7	43.2	31.2	44.4	0.33	1.00	0.19
IAA, RU, median (range)	8.6 (3.2-80.1)	8.6 (5.2-71.8)	9.5 (3.2-80.1)	10.2 (2.9-414.2)	0.73	0.59	0.90
IA-2A, %	63.8	67.6	59.4	76.0	**0.03**	0.32	**0.05**
IA-2A, RU, median (range)	100.1 (0.9-193.5)	100.6 (2.2-193.5)	99.5 (0.9-188.5)	103.4 (0.9-553.3)	0.42	0.66	0.46
GADA, %	69.6	75.7	62.5	67.4	0.80	0.37	0.70
GADA, RU, median (range)	49.8 (6.5-812.4)	49.8 (6.5-200.6)	62.2 (8.9-812.4)	37.7 (5.5-4,252.8)	0.19	0.68	0.12
ZnT8A, %	49.2	51.5	46.7	62.5	**0.04**	0.27	0.11
ZnT8A, RU, median (range)	6.1 (0.9-76.3)	4.2 (0.9-76.3)	16.3 (1.2-61.2)	6.4 (0.6-424.9)	0.70	0.20	**0.05**
Number of positive antibodies, median (mean)	3 (2.98)	4 (3.09)	3 (2.87)	4 (3.44)	**0.009**	0.29	**0.007**
HLA class II genetics							
DR3-DQ2/DR4-DQ8, %	20.0	10.8	30.3	21.7	0.85	0.16	0.33
DR3-DQ2/x*, %	29.6	13.5	47.1	14.4	**0.001**	1.00	**<0.001**
DR4-DQ8/y†, %	32.9	45.9	18.2	48.6	**0.01**	0.88	**0.001**
x*/y†, %	16.9	29.7	2.9	15.1	0.81	**0.03**	0.08
Protective genotype, %	9.9	16.2	2.9	3.2	**0.007**	**<0.001**	1.00
DR4-DQ8 positive, %	53.5	56.8	50.0	70.4	**0.003**	0.11	**0.02**
DR3-DQ2 positive, %	50.0	24.3	78.8	36.2	**0.03**	0.19	**<0.001**
DR3-DQ2 homozygote, %	11.3	2.7	20.6	2.9	**0.001**	1.00	**<0.001**
DR4-DQ8 homozygote, %	5.6	8.1	2.9	7.3	0.77	0.75	0.51

Children with family history of autoimmune diseases other than T1D

Demographics

Children with extended family history of AIDs other than T1D and children coming from autoimmune families were compared to children without any additional family history for AIDs. There were no gender differences between these groups (Table 16) or between sexes in the frequencies of having relatives AIDs in any specific relative groups. The frequency of reporting extended family members with these diseases was similar in all age groups; 37.2% in the age group of 0-5 years, 36.2% in the age group of 5-10 years, and 34.3% in the age group of 10-15 years, (P=0.52).

As expected, the children with positive family history for AID other than T1D and especially the children from autoimmune families came from larger families, i.e. the number of children in the family and birth order of the index child were higher, and the prevalence of being the only child lower in most groups compared (Table 16). Only when comparing children with mothers or fathers affected with AID other than T1D, this difference was not observed. In autoimmune families, also the age of the parents was higher.

Autoantibodies

In study II, higher levels of ICA and IA-2A were seen in the children with relatives affected by AID compared to the children with no family history of AID. In contrast, at the end of study, higher frequencies of IAA positivity were seen in children with family history of AIDs and lower frequencies of ICA positivity among children from autoimmune families (Table 16).

Children with mothers, fathers, or siblings affected with AID other than T1D or children with family history specifically for AIT compared to children with no family history for AIDs did not differ in terms of islet autoantibody profiles. The children with rheumatoid diseases in the extended family had higher ICA (44 vs. 40 JDF units, P=0.04) and IA-2A levels (109.1 vs. 101.9 RU, P=0.02) as well as were more often positive for IAA (51.4 vs. 42.1%, P=0.01).

HLA class II genetics

There were no differences in HLA class II genetics in the children with or without a positive family history of AID or children from autoimmune families (Table 16).

Children with mothers, fathers or siblings with AIDs other than T1D or chidren with relatives with AIT did not differ in terms of HLA class II genetics. However, the children with a positive family history for rheumatoid diseases carried less often the DR3-DQ2/nonDR4-DQ8 genotype (9.7 vs. 15.6%, P=0.03).

Metabolic decompensation at diagnosis

As discovered already in Study II, the children with or without family history for AIDs other than T1D did not differ in terms of markers of metabolic decompensation at diagnosis (Table 16). Children from autoimmune families, however, displayed a clearly milder metabolic decompensation at diagnosis (Table 16), which is probably due to the cases of familial T1D included in this group.

The children who had relatives with AIT had lower levels of β-hydroxybuturate (1.6 vs. 2.1 mmol/l, P=0.02) and higher pH (7.38 vs. 7.37, P=0.02) compared to the children without any family history of AID. Children with mothers, fathers or siblings with AIDs other than T1D, or children with family history of rheumatoid diseases did not differ in terms metabolic decompensation at T1D diagnosis.

Table 16. *Demographics, markers of metabolic decompensation at T1D diagnosis, islet autoantibodies, and HLA class II genetics in children with family history for autoimmune diseases (AID) other than T1D (I) or children from autoimmune families (II) compared to children without any family history for autoimmune diseases (III). *x≠DR4-DQ8 †y≠DR3-DQ2*

	I. Children with AID other than T1D in extended family (n=608)	II. Children from autoimmune families (T1D included) (n=270)	III. Children with no additional AID in extended family (n=1089)	I vs. III: P value	II vs. III: P value
Demographics					
Sex, male, %	56.6	55.6	57.6	0.73	0.60
Age at diagnosis, yrs, median (range)	8.13 (0.5-15.0)	7.85 (0.7-14.99)	8.32 (0.7-15.0)	0.43	0.29
Age of father at diagnosis, yrs, median (range)	40.6 (23.9-61.4)	41.0 (23.9-55.2)	39.7 (21.0-65.9)	0.24	**0.02**
Age of mother at diagnosis, yrs, median (range)	38.3 (21.1-52.4)	39.3 (21.1-53.2)	37.8 (18.7-56.2)	0.13	**0.01**
Only child, %	16.9	11.5	23.3	**0.003**	**<0.001**
Number of children in the family, median (mean)	2 (2.40)	2 (2.64)	2 (2.26)	**0.001**	**<0.001**
Birth order of the index, median (mean)	2 (1.85)	2 (2.05)	2 (1.72)	**0.002**	**<0.001**
Days from diagnosis at sample, median	5	5	5	0.91	0.27
Metabolic decompensation at diagnosis					
Blood glucose, mmol/l, median (range)	24.7 (3.2-68.1)	23.2 (3.2-63.7)	25.0 (4.9-92.7)	0.16	**<0.001**
Ketoacidosis, %	17.4	15.4	20.7	0.11	0.06
pH, median (range)	7.38 (6.88-7.52)	7.39 (6.92-7.54)	7.37 (6.84-7.52)	0.07	**0.001**
ß-hydroxybutyrate, mmol/l, median (range)	1.8 (0-15.2)	1.4 (0-17.4)	2.1 (0-16.2)	0.10	**<0.001**
Impaired consciousness, %	4.6	2.3	6.0	0.26	**0.03**
Duration of symptoms, days, median (range)	12 (0-377)	8.5 (0-143)	9 (0-314)	0.10	0.49
Weight loss, kg, median (range)	1.5 (0-20.0)	1.0 (0-13.0)	1.5 (0-17.0)	0.65	**0.003**
BMI (range)	15.4 (10.2-33.6)	15.7 (10.2-27.3)	15.5 (11.0-32.7)	0.23	0.42

Autoantibodies	I. Children with AID other than T1D in extended family (n=608)	II. Children from autoimmune families (T1D included) (n=270)	III. Children with no additional AID in extended family (n=1089)	I vs. III: P value	II vs. III: P value
ICA, %	93.9	88.3	93.5	0.85	**0.007**
ICA, JDFU, median (range)	44 (2.5-640)	40 (3-640)	40 (3-39,935)	0.07	0.38
IAA, %	47.5	43.4	42.1	**0.04**	0.76
IAA, RU, median (range)	9.7 (2.9-414.2)	12.4 (2.9-239.8)	10.3 (2.9-386.1)	0.27	0.26
IA-2A, %	78.8	72.3	75.0	0.10	0.41
IA-2A, RU, median (range)	105.2 (0.9-553.3)	103.9 (0.9-553.3)	101.9 (0.9-501.0)	0.08	0.28
GADA, %	69.1	71.9	67.2	0.48	0.18
GADA, RU, median (range)	42.1 (5.5-4,252.8)	41.7 (6.0-3,051.4)	36.2 (5.6-324.7)	0.91	0.88
ZnT8A, %	61.0	59.8	62.9	0.52	0.45
ZnT8A, RU, median (range)	7.2 (0.6-308.9)	5.9 (0.7-298.8)	6.4 (0.6-459.4)	0.42	0.19
Number of positive antibodies, median (mean)	4 (3.5)	4 (3.3)	4 (3.4)	0.35	0.36
HLA class II genetics					
DR3-DQ2/DR4-DQ8, %	22.9	23.1	20.7	0.33	0.44
DR3-DQ2/x*, %	14.1	15.3	15.6	0.45	0.98
DR4-DQ8/y†, %	47.7	47.6	46.8	0.78	0.88
x*/y†, %	15.3	13.9	16.6	0.53	0.33
Protective genotype, %	4.1	3.4	3.4	0.55	1.00
DR3-DQ2 positive, %	37.0	38.4	36.4	0.85	0.59
DR4-DQ8 positive, %	70.6	70.8	67.7	0.24	0.37
DR3-DQ2 homozygote, %	3.3	3.0	2.4	0.35	0.75
DR4-DQ8 homozygote, %	6.5	9.0	5.8	0.64	0.08

Characterizing type 1 diabetes associated with celiac disease

Demographics

By the end of data collection in Study III, 24 children of the 744 children included were diagnosed with CD. When including the additional children with known CD in the Register, there were 34 (1.5%) children with CD among the 2245 index children. Neither children with double diagnosis of T1D and CD (Table 15), nor the 36 children with

positive anti-tTG at T1D diagnosis (Table 1 in study III) differed from the children without CD or anti-tTG in terms of age at diagnosis or gender. Children with CD were less often the only child of the family. This is in accord with the fact that Study III with a search for silent CD had an inclusion criterium of at least one sibling with sample in the Sample repository.

The 79 anti-tTG positive first-degree relatives and the 67 first-degree relatives with CD (Table 2 in study III) were older and more often female than the relatives without anti-tTG or CD. There was also a tendency for having a diagnosis of an AID other than CD or T1D more frequently.

The median levels of total- or IgA-anti-tTG did not differ between the index children, mothers, fathers or siblings (data not shown), and neither did the proportion of those with high levels in either isotype among the anti-tTG positives (index children 44.4%, mothers 63.9%, fathers 55.6%, sibling 48.0%, P=0.38). Anti-tTG positivity not developing into CD during follow-up, however, was more common among the index children than first-degree relatives (63.9% vs. 39.2%, P=0.01). In general, those who developed CD had higher levels of both anti-tTG isotypes compared to those without CD; IgA-anti-tTG: 66.3 vs. 3.9 RU (P<0.001), total-anti-tTG: 10.7 vs. 4.6 RU (P<0.001). However, among those without CD but positive for anti-tTG, total-anti-tTG levels tended to be higher among index children (median in index children 6.9RU, mothers 4.0RU, fathers 4.4RU, siblings 3.4RU, P=0.049), but IgA-anti-tTG did not (index children 3.8RU, mothers 4.0RU, fathers 4.3RU, siblings 5.0RU, P=0.86). Anti-tTG positivity not leading to CD, was not limited to either total or IgA specific assay; 81.5% (44/54) of those positive for anti-tTG but without CD had total-anti-tTG and 70.4% (38/54) had IgA-anti-tTG. High total-anti-tTG levels were observed in four cases (7.4%) and high IgA-anti-tTG in 12 (14.8%). These proportions did not differ between index children, mothers, fathers or siblings. Accordingly, these findings do not support the hypothesis that lower levels of anti-tTG or antibody isotype profiles explained the higher frequency of benign anti-tTG positivity in index children.

Autoantibodies

Index children positive or negative for anti-tTG did not differ in terms of islet autoantibody profile (Table 1 in study III). The children with CD (Table 15 and Study III), however, tended to have lower levels of ZnT8A and less often IA-2A compared to children without anti-tTG or CD. Moreover, their overall number of positive islet cell autoantibodies was lower.

First-degree relatives with anti-tTG were more often positive for GADA, whereas there were no differences between relatives with CD and those without CD autoimmunity (Study III).

HLA class II genetics

The association of anti-tTG positivity and especially CD with HLA-DR3-DQ2 was clear-cut in both index children and first-degree relatives (Table 15 and Study III). OR for CD for this haplotype was 8.4 and for anti-tTG positivity 5.0. Of the children with both T1D and CD, 79% carried this haplotype, and of the first-degree relatives with CD, 73%. Homozygozity for this haplotype was observed in 21% of index children and 8% of the relatives with CD. As this population was patients or first-degree family members with T1D, the HLA-DR4-DQ8 was overrepresented in relation to other populations with CD. Only one index child and no relatives with CD, had neither of these two CD risk haplotypes.

Metabolic decompensation

There were no marked differences in markers of metabolic decompensation between children with anti-tTG or CD (Table 15 and Study III) and children without any additional autoimmunity; although there was some indication for lower weight loss or lower BMI in children with CD related autoimmunity.

Non-HLA genes and clustered autoimmunity

Association analyses

Of the 2245 children, 1780 were included in Study IV, but only 1745 of them passed the quality control step of having at least half of the tested polymorphisms genotyped successfully. Of these 1745 children, 60 (3.4%) had another AID in addition to T1D by the end of study. When association analyses for 41 SNPs were conducted and compared to children with T1D only, this phenotype associated nominally with two *RGS1* SNPs (Table 3 in study IV); rs2816316 (OR 1.88, P=0.006) and rs2984919 (OR 1.82, P=0.008). However, the minor allele associated for risk for additional autoimmunity in this analysis is the one associated with protection from T1D and CD in previous studies. Two SNPs;

rs2476601 (*PTPN22*) and rs4763879 (*CD69*) associated with having first-degree family members with T1D (Table 17). The effect direction for both of these SNPs (minor allele predisposing) is the same as reported earlier for T1D and other AIDs. These same SNPs were not associated with having first- and/or second-degree relatives with T1D, however. Instead, the minor allele for *TNFAIP3* associated with risk of familial T1D. This is risk allele for other AIDs, but the risk allele for T1D is not known (Table 1 in study IV). SNP rs11711054 (*CCR3-CCR5*) associated with coming from an autoimmune family (OR 0.69, Table 3 in study IV). Correcting for multiple tests by the FDR procedure removed statistical significance from all these findings.

Multivariate analyses

A model including DR4-DQ8 was better at explaining multiple AIDs in children with T1D compared to the model with only confounding factors (Table 4 in study IV). On the contrary, the HLA haplotypes were determined unuseful in explaining clustering in autoimmune families. Non-HLA SNPs, however, improved the models for both phenotypes in comparison to models including only confounding factors (Table 5 in study IV). The SNPs associated with having multiple AIDs were neuropilin 1 (*NRP1*, rs2666236), *RGS1* (rs2816316), *CD69* (rs4763879), and *FUT2* (601338). The SNP associated with autoimmune families was *CCR3-CCR5* region (rs11711054).

Effects of combined number of risk alleles

Combined numbers of risk alleles (0, 1, or 2 per SNP) were compared between the phenotypes. For familial T1D, 19 SNPs associated with T1D earlier in the same population were included (number of risk alleles weighted by their OR for T1D was also used). For phenotype of clustered AIDs other than T1D, the figure was 25 SNPs known to be associated with other AID from literature. For autoimmune families, a combination of 28 risk SNPs for T1D and other AIDs were calculated. None of these mean/median numbers of risk alleles differed between any of the phenotypes (Supplemental Figure 1 in study IV)

Table 17. *Results of association analyses for 41 SNPs in familial T1D compared to children with T1D only and without any family history for AIDs. Odds ratios (OR) with 95% confidence intervals (CI) for minor alleles are shown.*

CHR	SNP	Gene	Minor/ major allele	MAF	T1D in first-degree relatives (n=221)				T1D in first and/or second-degree relatives (n=398)			
					OR	95CI	*P* value	FDR *P* value	OR	95CI	*P* value	FDR *P* value
1	rs630115	*LOC646538*	A/G	0.33	0.95	0.76-1.19	0.67		0.97	0.81-1.17	0.77	
1	rs2476601	*PTPN22*	A/G	0.22	**1.30**	**1.01-1.66**	**0.04**	0.49	1.15	0.94-1.41	0.18	
1	rs2816316	*RGS1*	G/T	0.13	0.91	0.65-1.26	0.56		0.86	0.66-1.12	0.27	
1	rs2984919	*RGS1*	A/T	0.13	0.94	0.68-1.29	0.69		0.87	0.67-1.13	0.28	
1	rs12061474	*PIK3C2B*	T/C	0.19	1.25	0.96-1.61	0.10		1.15	0.93-1.42	0.21	
1	rs3024505	*IL10*	T/C	0.14	0.87	0.63-1.21	0.41		0.84	0.65-1.09	0.20	
2	rs6546909	*DQX1*	T/A	0.16	0.98	0.74-1.30	0.91		0.81	0.64-1.03	0.08	
2	rs9653442	*LOC150577*	C/T	0.45	1.03	0.84-1.28	0.76		1.03	0.86-1.22	0.78	
2	rs917997	*IL18RAP*	A/G	0.18	1.02	0.78-1.34	0.87		1.03	0.83-1.29	0.77	
2	rs2111485	*IFIH1*	A/G	0.38	1.03	0.83-1.28	0.81		1.07	0.90-1.27	0.47	
2	rs1990760	*IFIH1*	C/T	0.37	1.01	0.82-1.26	0.90		1.01	0.85-1.21	0.90	
2	rs7574865	*STAT4*	T/G	0.24	1.12	0.87-1.44	0.37		1.08	0.88-1.32	0.47	
2	rs3087243	*CTLA4*	A/G	0.31	0.90	0.71-1.14	0.39		0.91	0.76-1.10	0.35	
2	rs11571297	*CTLA4*	G/A	0.34	0.91	0.73-1.14	0.42		0.93	0.78-1.12	0.45	
3	rs11711054	*CCR3-CCR5*	G/A	0.29	0.88	0.69-1.11	0.28		0.86	0.71-1.05	0.13	
3	rs333	*CCR5-Delta32 delet.*	T/A	0.13	1.08	0.79-1.47	0.63		1.13	0.88-1.45	0.33	
4	rs17388568	*ADAD1*	A/G	0.42	0.98	0.79-1.22	0.87		0.89	0.75-1.06	0.20	
5	rs7719828	*LOC645261*	T/C	0.29	1.01	0.80-1.28	0.91		1.01	0.84-1.22	0.88	
6	rs3757247	*BACH2*	A/G	0.38	0.98	0.79-1.22	0.87		0.98	0.82-1.17	0.83	
6	rs6920220	*TNFAIP3*	A/G	0.21	1.28	0.99-1.65	0.06		**1.26**	**1.02-1.55**	**0.03**	0.95
10	rs12722495	*IL2RA*	G/A	0.05	0.99	0.62-1.59	0.97		1.00	0.69-1.46	0.99	
10	rs2104286	*IL2RA*	G/A	0.19	0.97	0.74-1.26	0.80		0.92	0.74-1.15	0.48	
10	rs4749955	*IL2RA*	C/T	0.46	0.97	0.78-1.20	0.74		1.03	0.87-1.23	0.70	
10	rs11258747	*PRKCQ*	T/G	0.27	1.02	0.80-1.30	0.88		0.95	0.79-1.16	0.64	
10	rs2666236	*NRP1*	T/C	0.38	1.07	0.86-1.33	0.56		0.97	0.81-1.16	0.77	
11	rs689	*INS*	T/A	0.12	0.78	0.54-1.12	0.17		0.97	0.74-1.27	0.81	
12	rs3764021	*CLEC2D*	G/A	0.48	1.23	0.99-1.52	0.06		1.15	0.97-1.36	0.12	
12	rs4763879	*CD69*	A/G	0.36	**1.26**	**1.01-1.57**	**0.04**	0.49	1.17	0.98-1.39	0.09	
12	rs1701704	*IKZF4*	C/A	0.37	1.04	0.83-1.29	0.76		1.00	0.84-1.19	0.98	
12	rs4646536	*CYP27B1*	C/T	0.34	0.95	0.76-1.19	0.64		1.01	0.84-1.21	0.90	
12	rs2292239	*ERBB3*	A/C	0.34	1.10	0.87-1.37	0.43		1.03	0.86-1.24	0.75	
12	rs3184504	*SH2B3*	T/C	0.44	0.90	0.72-1.12	0.33		0.96	0.81-1.14	0.66	
12	rs17696736	*C12orf30=NAA25*	G/A	0.42	0.94	0.75-1.17	0.57		0.92	0.77-1.10	0.39	
13	rs9585056	*GPR183*	C/T	0.26	1.04	0.81-1.32	0.78		0.99	0.82-1.21	0.96	
15	rs3825932	*CTSH*	T/C	0.37	1.12	0.90-1.39	0.31		1.02	0.86-1.22	0.83	
16	rs12708716	*CLEC16A*	G/A	0.30	0.97	0.77-1.23	0.83		1.03	0.85-1.24	0.75	
16	rs2903692	*CLEC16A*	A/G	0.28	0.94	0.74-1.20	0.62		0.99	0.82-1.20	0.91	
18	rs45450798	*PTPN2*	C/G	0.18	1.06	0.81-1.39	0.67		1.00	0.80-1.25	0.99	
18	rs763361	*CD226*	T/C	0.46	1.02	0.82-1.26	0.86		1.05	0.88-1.25	0.58	
19	rs601338	*FUT2*	A/G	0.40	1.24	1.00-1.54	0.05	0.49	1.10	0.92-1.31	0.30	
19	rs602662	*FUT2*	A/G	0.44	1.21	0.98-1.50	0.078		1.08	0.91-1.28	0.39	

Discussion

This study aimed at collecting current data on frequencies of additional autoimmunity in children with newly diagnosed T1D and in their relatives, characterizing the diabetes phenotype of these children according to their autoimmune history for AIDs in general and for CD specifically, as well as delineating the effect of non-HLA genes on clustering autoimmunity. At least 3.2% of the children with T1D developed additional AIDs, 23% reported first- and/or second-degree relatives with T1D, and over a third, relatives with other AIDs. Differencies especially in genetic etiology were discovered behind different phenotypes of clustering autoimmunity

Frequency of AIDs in T1D

Our results are in line with the notion that most additional AIDs develop after the diagnosis of T1D; only 1.6% of the children had another AID at diagnosis whereas a prevalence of 3.2% was calculated later even without systematic follow-up for all the children. These figures are in accord with those reported previously in the literature, although lower than reports that have systematically screened for different autoantibodies or included longer follow-up. In accordance with previous reports, CD and AIT were the most prevalent conditions. For other AIDs the figures presented in the thesis are based on self-reporting of known diagnoses, but CD was screened by anti-tTG. The prevalence of celiac disease among systematically screened children with T1D in our study was 3.2% (1.5% of the total cohort of 2245 children), which is close to the median 4% found in literature reviews [197, 198, 273]. The variation between populations is wide, however, and for example in the neighboring Sweden the prevalence of CD among T1D patients is 10% [182, 229]. In general, the prevalence of the high CD risk haplotype, DR3-DQ2, among Finnish patients with T1D is relatively low; the prevalence of this haplotype was 35.3% in our study compared to 50-60% reported in other populations [52, 274-276].

The frequency of familial T1D was 23%; 13.6% in first-degree and 12.5% in second-degree relatives. The proportion of familial T1D has remained similar from the 1990's when Veijola et al. reported the prevalence of first-degree familial T1D of 15.1% [145]. More than a third of the children reported extended family members with AIDs other than T1D, which is in accordance with previous literature. As expected these diseases were more commonly reported in second-degree relatives, who are generally older and thus more susceptible to most AIDs, for example rheumatoid diseases. Accordingly, AIT and rheumatoid diseases were the most commonly reported diseases among the relatives.

In study III, the prevalence of CD in the first-degree relatives (2.4%) was the same as that reported for Finnish adult general population [227], suggesting that the risk of CD is might not be elevated in first-degree relatives of Finnish children with type 1 diabetes. Similar reports have been published earlier with siblings of Finnish T1D patients [277].

Gender differences

Some interesting gender differences were evident. In general, T1D is an exceptional AID since there is no excess of female patients. Additionally, there are peculiar gender differences in the inheritance of the disease; T1D is more common in fathers than in mothers, and accordingly our prevalence in fathers (6%) was double that in mothers (3%). In epidemiological studies of T1D, preferential transmission from fathers to daughters and from mothers to sons has been reported, although our results do not support this. In contrast, our data supports a similar phenomenon from grandparents to grandchildren; maternal grandparents transmitted the disease to boys and paternal grandparents to girls. There were differences in HLA genetics to support this finding as well, although the actual mechanism for this phenomenon remains unknown. Interestingly, similar gender patterns have been described in MS, which is more prevalent in females, and the risk of the offspring, especially daughters, is higher for affected mothers. In fact, the risk mediating HLA-DRB1*15 has been shown to be preferentially transmitted from mothers [278, 279]. In female patients, the risk is additionally influenced by gender of affected second-degree relative (aunt-uncle). Also, the risk carried by HLA-DRB1*15 is significantly greater in families with affected second-degree relatives compared to families with only affected first-degree relatives. Suggested reasons behind these changes are gene-environment interactions and epigenetic changes [215].

Despite fathers having T1D more often than mothers, affected maternal and paternal second-degree relatives were reported equally often, as were affected grandfathers and grandmothers. In contrast, for AIDs other than T1D, mothers were more often affected than fathers, which is in line with autoimmunity associating with female gender in general. In keeping with this, affected maternal second-degree relatives were reported more often than paternal second-degree relatives. As these relatives are of both sexes, i.e. maternal grandfathers and uncles in addition to grandmothers and aunts, this difference cannot be attributed to gender. The difference could arise from recollection bias as mothers may be more aware of the diseases of their family members. Furthermore, mothers might be the primary parent accompanying the child to the diabetes visits and be the parent responsible for filling out the questionnaire for the Register. Such a recollection bias could be assumed to affect also reporting of T1D in paternal vs maternal

relatives, but this was not evident in this dataset. Other possibility is that AIDs are preferentially transmitted from the maternal side of the family by some unknown mechanism. Interestingly, this has been described in MS, with the disease being preferentially transmitted to offspring from maternal compared to paternal aunts/uncles [280]. Reasons for such pattern of transmission are unknown.

As AIDs are usually more common among females, more girls with an additional AID in combination with T1D could have been expected. Indeed, a tendency for this was evident with 4.0% of girls and 2.6% of boys having additional AIDs, but the difference did not reach statistical significance (P=0.09) possibly due to inadequate power. Study III focused on CD, and a unique gender distribution was discovered; contrary to the usual female predominance in CD, both sexes were equally often affected. Among the first-degree relatives there was the usual female majority, however.

Characterizing type 1 diabetes associated with clustered autoimmunity

We hypothesized that the children with clustered autoimmunity would form a separate subgroup in terms of their diabetes phenotype and genotype. We assumed that genetic risk for autoimmunity would be higher in families with clustered AID, i.e. gene regions known to be associated with a broad spectrum of autoimmune disorders (e.g. HLA-DR3-DQ2, *PTPN22*, *CTLA-4*) were expected to be overrepresented. We also expected a broader humoral autoimmune reaction in children with wide range of autoimmune phenotypes.

The differences evident in humoral autoimmune profile were the opposite from our hypothesis, however. More specifically, children with familial T1D, children from autoimmune families, and children with additional AIDs (other than CD) were less often positive for ICA. Results in the same direction (lower frequency of autoantibody positivity) were seen for children with additional AIDs for IA-2A and ZnT8A. Moreover, these children, especially children who already had or later developed CD, had a lower number of positive islet autoantibody responses at diagnosis of T1D. These differences might be due to differential genetic background. For children with T1D and CD, the differences are readily explained by the high prevalence of DR3-DQ2 haplotype. DR3-DQ2 has been associated mostly with GADA and low/less prevalent IAA and IA-2A [20, 24]. ICA are associated with DR4-DQ8 [20] and thus a higher, not lower as observed, prevalence could have been expected in familial T1D. Consequently, these findings are difficult to explain. Some significant P values could also arise by chance. Nevertheless, as GADA are associated to general propensity to autoimmunity, an increased prevalence and high titers would be expected for children with clustered autoimmunity. Accordingly,

high GADA levels were seen in children with multiple AIDs in study II after adjustement for age at T1D onset (Table 1 in Study II). Also, relatives positive for anti-tTG in study III had GADA more often. In the pooled results, however, differences in GADA prevalences or titers did not reach statistical significance.

Familial T1D was characterized by better clinical situation at diagnosis of T1D even when the affected relatives were second-degree relatives. In Finland, these relatives usually do not live in the same household as the immediate family. These results are encouraging in the sense that the information of the disease seems to spread even from relatives outside the immediate family allowing earlier recognition of the disease. As the prevalence of the sporadic disease was less than 80% when second-degree relatives were considered, the finding is promising in terms of attempts to prevent diabetic ketoacidosis. On the other hand, the prevalences of ketoacidosis (10% of first-degree familial cases and 11% of second-degree familial cases) are still high and there is room for improvement of early recognition of T1D.

Having family members with AIDs other than T1D did not affect the markers of the metabolic decompensation at diagnosis. However, if the child had an AID diagnosed already before diagnosis of T1D (study II), the clinical situation was better than children with T1D alone. Perhaps these children are being followed-up for their disease and are therefore screened or their symptoms are recognized earlier than for children without such diseases. However, the duration of symptoms was in fact longer, not shorter, contradicting the idea of earlier diagnosis behind the milder metabolic decompensation. One possible explanation would be recollection bias; it is possible that families with prior experience of AIDs think that they should have been able to recognize the very early symptoms and accordingly estimate longer duration of symptoms than sporadic cases. It is also possible that the pathogenesis of T1D is different in these children and perhaps the course of development of T1D is slower or milder leading to less symptoms at diagnosis, although our data does not support this. If the children diagnosed with additional AIDs later during follow-up were included in the analysis, the differences in markers of metabolic decompensation were less pronounced. This finding would support the hypothesis of children with pre-existing conditions being more closely monitored for additional diseases or that the possible slower disease progression is limited to children diagnosed with additional AIDs before T1D diagnosis.

We hypothesized that children with additional AIDs or family history for such diseases would have an increased HLA mediated risk. For familial T1D this has been described in Finnish population as higher prevalences of risk genotypes DR3-DQ2/DR4-DQ8 and DR4-DQ8/nonDR3-DQ2 as well as decreased prevalences of low risk or

protective genotypes among children with first-degree relatives with T1D [145]. In study I, with information on family history of T1D limited only to the situation at diagnosis of the index case, we saw differences only in one haplotype; HLA-DR4-DQ8. However, with a larger sample size, data on additional T1D diagnoses in the family, and with a different comparison group more differences became apparent; children with familial T1D had more often one or two copies of the DR4-DQ8 haplotype and less often a genotype without either of the risk haplotypes. Children with family history of AIDs other than T1D or children from autoimmune families did not differ in terms of HLA class II genetics, but family history of CD associated with DR3-DQ2 in the index child.

Children with additional AIDs differed in their HLA genetic profile; children with CD related autoimmunity had the DR3-DQ2 haplotype or related genotypes whereas children with AIDs other than CD had more often genotypes without either of the T1D risk haplotypes or genotypes protective for T1D. Accordingly, it seems that children with multiple AIDs are prone to develop T1D with weaker HLA-mediated risk and even with HLA-mediated protective genotypes. This provokes interesting questions about the pathogenesis of T1D in these children. They might have some other genetic loci predisposing them to T1D or autoimmunity in general, or some strong environmental factors leading to break-up of self-tolerance. Possibly the loss of tolerance is first developed against other tissues and later spread to β-cells. In study IV, *RGS1* was associated with other AIDs in children with T1D. Half of this group of children had CD, however, and this could account for the association, as *RGS1* is a known CD risk SNP [86]. Nevertheless, this group of children with AIDs other than CD is an interesting group for further studies on clustering autoimmunity.

Together, these results indicate that HLA class II genetics plays a role in familial clustering of T1D and CD; HLA-DR4-DQ8 in familial T1D and DR3-DQ2 in T1D with CD autoimmunity. Differential disease pathways have been suggested for these haplotypes; DR4-DQ8 associates with IAA and DR3-DQ2 with GADA as the first autoantibody [25-27]. Accordingly, familial T1D could arise throught the pathogenetic route of IAA and T1D in the context of CD through GADA. We can not analyse this directly, however, as the Register does not include data from the prediabetic period.

The differences in HLA genotype/haplotype profiles for familial T1D were modest with a sample size of 324 in study I, whereas at the end of study with a sample of 522 familial T1D cases, more differences could be seen. In 1996, the differences in HLA class II genetics were evident already with a sample size of 121 familial cases and the prevalence of the highest risk HLA class II genotype DR4-DQ8/DR3-DQ2 among familial T1D cases was higher than in the current study: 33% vs. 23%. Comparison of

the other haplotypes and genotypes is difficult since the risk grouping of HLA class II genetics has changed. This decrease in the prevalence of high risk genotype may indicate that the effect size of HLA class II genetics on familial clustering has decreased within the last decades with more pressure for this clustering from environmental risk factors. Similar phenomenon has been seen in T1D development; patients diagnosed with T1D recently have a lower HLA mediated genetic risk for T1D than patients diagnosed decades ago, suggesting that the need for genetic susceptibily to develop the disease has decreased as the environmental pressure for disease development has increased [281].

As multiple other genetic loci have been associated with AIDs in addition to HLA, we tested the involvement of these in clustering of autoimmunity. However, the power of the study was very limited and thus convincing results were difficult to acquire. Multivariate analysis, however, indicated an overall effect of non-HLA SNPs on clustering of AIDs in children with multiple AIDs and in autoimmune families. Some candidate genes were discovered, but need validation in other studies. *RGS1* and *CCR2-CCR5* alleles predisposing to T1D seemed to protect from clustering autoimmunity, which could just reflect a decreased genetic risk load to T1D in clustered autoimmunity. Nevertheless, *PTPN22* as a risk gene for familial T1D is a credible finding as this is a gene with relatively large effect size on T1D and has a function in immune reactions. However, the association was only seen in children with first-degree relatives with T1D, not if affected second-degree relatives were included. Interestingly, no association with *INS* was evident although this is a strong risk SNP and unique for T1D. Nonetheless, it is important to keep in mind, that despite the limited power of the study, large effect sizes of these SNPs on clustering of AIDs would have been discovered. Our findings are in line with the notion that the genetic predisposition to familial T1D is mainly attributed to HLA class II genes and not genes outside the HLA region [74], as observed by smaller effect sizes for non-HLA loci in families with affected siblings than from sporadic cases [66]. Additionally, these results emphasize the role of other factors in clustering of autoimmunity. Indeed, environmental risk factors cluster in families in addition to genetic factors, and can thus explain the increased risk in family members compared to general population. Shared microbiota, intrafamilial spread of enteroviruses [282], and nutrition are examples of suggested T1D risk factors that are shared by family members.

We observed differences in the family demographics between different phenotypes of clustered autoimmunity. These are mostly direct results of the definitions used. For example the children with positive family history for AID other than T1D and the children from autoimmune families came from larger families, since in larger families there are more family members susceptible to AIDs. Also, naturally the children who

were the only child of the family were younger at diagnosis since families with very young children have not had the time to have more children.

Transient anti-tTG at diagnosis of T1D?

Children with both T1D and CD had some unique features compared to children with only one of the diseases. These children had the HLA class II haplotype DR3-DQ2 more often than children with T1D only (79% vs. 36%) and less often than usually reported for children with CD (90%). They had a lower number of islet cell autoantibodies. This could be due to the difference in HLA DR3-DQ2 prevalence, or a different – perhaps less aggressive – β-cell destruction in children with ongoing celiac autoimmunity or in those who will develop this autoimmunity later. Also, both boys and girls with T1D equally often developed CD in contrast to the usual female predominance evident both in children and adults with CD only.

There was a striking difference between index children and first-degree relatives in the proportion of those who were anti-tTG positive in the Register sample and were diagnosed with CD; only 36% of the index children and 60% of the relatives positive for anti-tTG had CD. The index children did not have lower levels of anti-tTG, but they were less often positive for EMA. It is possible that some of these children will develop CD in the future, although information from the hospitals in charge of the follow-up indicated that most of these children were negative for CD related autoantibodies during follow-up. First-degree relatives of these children were also older and might therefore have developed CD more often. Also, they might have developed CD autoimmunity long before the sample was taken and suffered from silent CD. Nevertheless, these factors do not fully explain why children with newly diagnosed T1D were anti-tTG positive so often but still did not develop CD. A plausible explanation would be transient anti-tTG positivity related to the time of diagnosis of T1D in the index children. There are previous reports of transient anti-tTG positivity in 50% of children without T1D but genetically at risk for CD [283] and false positives in anti-tTG testing are generally described more often in patients with other AIDs [200]. In a prospective Italian study from T1D diagnosis onwards, 40% of those positive for anti-tTG showed fluctuating levels during follow-up and 28% became negative. The normalization of anti-tTG was associated with being EMA negative, low levels of anti-tTG, being asymptomatic, and older age at CD autoantibody development [258]. In Israel, spontaneous normalization of anti-tTG occurred in 35% of pediatric T1D patients [242], and the authors proposed a 12-month serological follow-up of cases with low anti-tTG levels before biopsy. In other reports the proportion of transient CD-related autoimmunity has ranged from 2.3% to 55% [229,

237, 284]. The proposed mechanisms of transient autoantibodies have been associated with active β-cell destruction [247] or abnormal immunoglobulin levels (elevated IgA) [237, 285] at diagnosis of T1D. Studies with systematic screening of CD related autoantibodies around the time of diagnosis of T1D should be carried out to clarify whether this time is especially vulnerable to transient autoimmunity.

Strengths and limitations of the study

This study is a relatively large population-based observation study of children diagnosed with T1D in a country of highest incidence for this disease in the world. Accordingly, the Finnish population is especially interesting for the study of diabetes and we had a unique resource in the Finnish Pediatric Diabetes Register with its coverage of over 90% of all children diagnosed with T1D in the country since 2002. We were, however, forced to use only the children who participated in the Sample Repository in our studies, since we wanted to compare genetics and humoral autoimmunity profiles. This might cause some selection bias, since there seemed to be a tendency for children with family history of T1D to provide samples in addition to the Register questionnaire more frequently. It is reasonable to assume, that families who have many members with T1D or other AIDs, might be more motivated to participate in studies of these diseases compared to children without such a family history. This bias might somewhat overestimate our figures of clustering autoimmunity, but systematic bias in comparison of familial and sporadic cases seems unlikely. Moreover, our study was completely unable to aquire data on those who did not participate in the Register at all.

In addition, in ascertaining the autoimmune diagnosis of the children and their family members were relied on information provided by the families on a questionnaire. A more reliable way of ascertainment would have been to screen for autoantibodies for different diseases, or ascertainment from patient records or national registers. These methods would have been too costly and laborsome and, accordingly, would have limited the sample size. It is likely that there is both under- and overreporting of AIDs in the questionnaire although help of diabetes nurse or doctor was encouraged in the filling of the questionnaire. Thus, we cannot guarantee the autoimmune origin of all diagnoses reported. However, these shortcomings should not lead to any systematic bias in either direction as both under and overreporting are expected. Not all families want to disclose all information on the family in the questionnaire. As a result, not all siblings and diseases of the relatives are reported. Also, detailed information on missing information of AIDs of specific relative groups is lacking and accordingly, we assumed that all fields left blank

in the questionnaire represented cases without AIDs. Thus, our figures on frequencies of having AIDs in relatives might be slight underestimations.

The Register is a cross-sectional study without systematic follow-up of the children. Thus, most information included in the study is from a single time-point. We have no information from the preclinical phase of T1D development, and thus cannot study for example the temporal development of islet autoantibodies. The follow-up data for CD and AIT is acquired by alternative follow-up methods and only for a subset of the families. Therefore, the prevalences of AIDs reported are underestimations, and CD and AIT are overrepresented in relation to other AIDs. However, compared to original studies, increasing the sample size and adding all the available follow-up data for the pooled analysis, increased the power of the study to detect differences between familial and sporadic cases (i.e. additional genetic differences in familial T1D described here compared to study I). These limitations in ascertainment of cases with AIDs might dilute the differences detectable between cases with and without addional autoimmunity.

In studies on non-HLA genetics, the small sample size leading to inadequate power was the major limitation. Despite being based on a large nationwide register, the numbers of cases in the phenotype groups are not large enough, which emphasizes the need for large international studies. In addition, the SNPs used in the analyses were primarily chosen for analysis for T1D risk association. The comparisons of phenotypes of clustering of other AIDs might have required a different set of SNPs. Accordingly, our results on non-HLA associations require validation in other studies.

Conclusions

1. At diagnosis of T1D, 1.6% of Finnish children have additional AIDs. This proportion increases to at least 3.2% after median of eight years of follow-up. The children with CD and T1D carry the HLA-DR3-DQ2 haplotype, while children with AIDs other than CD carry neutral or protective T1D HLA-genotypes conspicuously often. Also, non-HLA loci contribute to the clustering of AIDs, and *RGS1* (rs2816316) is a candidate gene in children with multiple AIDs. These children have some differences in islet autoantibodies, possibly due to the differences in HLA class II genetics. The duration of T1D symptoms is longer for children with additional AIDs, and additional evidence suggests milder onset of T1D in such children.

2. More than 20% of children with recent-onset T1D in Finland come from extended families with T1D. Affected first- and second-degree relatives are reported equally often (~12%), whereas fathers are affected twice as frequently as mothers (6 vs. 3%). Girls have paternal and boys maternal second-degree relatives affected by T1D more often. Familial T1D is characterized by an increased prevalence of the HLA-DR4-DQ8 haplotype but differences in islet autoantibodies are modest. Increased awareness of the disease in the extended family – even if only second-degree relatives are affected – leads to a significantly better metabolic status at diagnosis.

3. Over a third of children with T1D in Finland have extended family members with AIDs other than T1D. Mothers are affected more than twice as often as fathers (10 vs. 4%). Children with extended family history for other AIDs come from larger families, but differences in HLA class II genetics, islet autoantibodies or metabolic decompensation at diagnosis are only modest. Non-HLA loci, in contrast, contribute to this autoimmune clustering, and rs11711054 (*CCR3-CCR5*) is a candidate gene for familial autoimmunity.

4. According to systematic screening data of Study III, of the Finnish index children with newly diagnosed T1D, 5% have anti-tTG and of their relatives 3%. The prevalence of CD was 3.2% for index children (1.5% for the total cohort of 2245) and 2.5% for relatives. Relatives with CD related autoimmunity are older and

more often female. HLA-DR3-DQ2 is related to CD autoimmunity in index children and relatives. Transient anti-tTG not developing to CD seems more common among children with T1D compared to their first-degree relatives.

In summary, differencies in genetic etiology were discovered behind different phenotypes of clustering autoimmunity, but clear-cut associations for e.g. certain islet autoantibodies were not evident. Prospective studies with follow-up before and after T1D diagnosis would provide data for example on the timing of islet autoimmunity and the appearance of additional AIDs, but such studies would require very large cohorts to have adequate power. The observed candidate non-HLA associations and transient anti-tTG in children with T1D require confirmation in future studies.

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